The immunomodulatory properties of Vitamin D in vitro and in vivo

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Abstract

Asthma is a chronic inflammatory disease of the airways. The current cornerstone for asthma treatment is glucocorticoids (steroids), and the majority of patients respond to steroids with an improvement in lung function (Steroid Sensitive; SS). However a proportion of asthmatics do not respond to steroids with improvement in lung function (Steroid Resistant; SR), who represent those patients most at risk. Epidemiological studies demonstrate that asthma severity and poor responsiveness to treatment, is associated with vitamin D insufficiency and deficiency. Vitamin D is an immunomodulatory molecule which induces the synthesis of antimicrobial peptides and anti-inflammatory molecules, and inhibits pro-inflammatory cytokine synthesis. The overall goal of this work was to investigate immunological differences between SS and SR asthma patients, and effects of vitamin D that may explain the epidemiological observations.

Peripheral blood from a clinically well-characterized cohort of SS (n=14; mean improvement in lung function or FEV_1 post 2-weeks oral prednisolone 16.1%) and SR (n=23; no improvement in FEV_1) asthmatics was studied by *ex vivo* flow cytometry. Major findings included evidence for significantly higher frequency of myeloid dendritic cells, and lower frequency of Foxp3+ CD4+ T cells in the peripheral blood of SR as compared to SS asthmatics. The accepted measure of vitamin D status is serum 25-hydroxyvitamin D3, 25(OH)D, and the severe asthmatics had significantly lower levels of serum 25(OH)D as compared to healthy controls. A significant positive correlation between the number of Foxp3+ T cells in the peripheral blood and serum 25(OH)D was observed.

Following culture of PBMCs with anti-CD3 activation it was found that severe asthmatics synthesised significantly higher IL-17A levels than healthy controls, which was most striking in the SR patients. The steroid Dexamethasone (Dex) enhanced IL-

17A production in culture, whereas the active form of vitamin D 1,25-hydroxyvitamin D3 ($1,25(OH)_2D3$) significantly reduced IL-17A production in a Dex-independent manner. This work proposed that IL-17A inhibition by $1,25(OH)_2D3$ may be partially due to a CD39-dependent mechanism.

In vivo evidence for a positive association between serum 25(OH)D and Foxp3+Treg numbers was further investigated *in vitro*. High concentrations of $1,25(OH)_2D3$ (10^{-6}M) increased the frequency of Foxp3+ T cells in culture through two main mechanisms: the induction of IL-2, on which Foxp3+Treg are dependent, as well as less inhibition of Foxp3+ over Foxp3- T cell proliferation. Lower, and likely more physiological concentrations of $1,25(OH)_2D3$ (10^{-7}M), which are known to enhance IL-10 synthesis, failed to significantly increase the frequency of Foxp3+ CD4+ T cells. However upon modulation of the cytokine environment to one of low IL-10 and high TGFβ, this concentration of $1,25(OH)_2D3$ significantly increased Foxp3+ Treg frequency.

Collectively this data highlights additional immunoregulatory properties of $1,25(OH)_2D3$ including induction of anti-inflammatory cytokines such as IL-10 and TGFβ, inhibition of pro-inflammatory cytokines such as IL-17A and IL-22 as well as the direct induction of Foxp3+ Tregs. In conclusion evidence for a number of immunomodulatory effects of $1,25(OH)_2D3$ exists that may help to reduce uncontrolled inflammatory responses associated with severe asthma.

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Abbreviations

1,25-$(OH)_2$D3	-	1,25-Dihydroxyvitamin D3
25(OH)D	-	25,hydroxyvitamin D
A467	-	Alexa Fluor 647
ab	-	antibody
AP-1	-	activator protein 1
APC	-	allophycocyanin
APCs	-	antigen presenting cells
ATP	-	Adenosine Triphosphate
BAL	-	bronchoalveolar lavage
BDCA	-	blood dendritic cell antigen
Bregs	-	B regulatory cells
BTK	-	Bruton’s typrosine kinase
C	-	Celsius
CBA	-	Cytometric Bead Array
CD	-	Cluster of differentiation
CNS	-	Conserved Non-coding Sequences
CTLA4	-	Cytotoxic T Lymphocyte Associated Protein 4
DAMPs	-	Danger Associated Molecular Patterns
DC	-	Dendritic Cell

Dex	-	Dexamethasone
dNTPs	-	Deoxynucleotide triphosphates
EAE	-	Experimental Autoimmune Encephalomyelitis
EdU	-	5-ethynyl-2'-deoxyuridine
ELISA	-	Enzyme-Linked ImmunoSorbent Assay
ERK	-	extracellular-signal-regulated kinase
FACS	-	Fluorescence-Activated Cell Sorting
FEV_1	-	forced expired volume in 1 second
FITC	-	fluorescein isothiocyanate
FoxP3	-	Forkhead box P3
GARP	-	glycoprotein A repetitions predominant
GATA-3	-	GATA binding protein 3
GITR Related Protein	-	Glucocorticoid-Induced Tumour necrosis Factor Receptor
GM-CSF	-	Granulocyte-macrophage colony-stimulating factor
GR	-	glucocorticoid receptor
GRE	-	Glucocorticoid Response Element
HATs	-	Histone acetyl transferases
HBSS	-	Hanks balanced salt solution
HDAC	-	Histone deacetylase

IFN	-	Interferon
IL	-	Interleukin
IL-10R	-	IL-10 Receptor
ILC	-	Innate Lymphoid cells
ILT3	-	Immunolglobulin-like transcript 3
iTreg	-	induced Treg
JNK	-	JUN amino-terminal kinase
L	-	Litre
LAG3	-	Lymphocyte Activating Gene 3
LAP	-	Latency Associated Protein
m	-	milli
M	-	Molar (moles per L)
MAPK	-	mitogen-activated protein kinases
min(s)	-	minute(s)
MMPs	-	Matrix metalloproteinases
MS	-	Multiple Sclerosis
MyD88	-	Myeloid differentiation primary response protein 88
n	-	nano
Na_2CO_3	-	Sodium carbonate
$NaHCO_3$	-	Sodium bicarbonate

NFκB	-	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	-	Natural Killer
NODs	-	Nod-like Receptors
NOD2	-	nucleotide-binding oligomerization domain containing 2
nTreg	-	natural T regulatory
o	-	degrees
OD	-	Optical density
PAMPs	-	Pathogen Associated Molecular Patterns
PBMCs	-	Peripheral blood mononuclear cells
PD-1	-	Programmed death 1
PDL-1	-	Programmed death ligand 1
PBS	-	Phosphate-buffered saline
PE	-	phycoerythrin
PerCp	-	peridinin-chlorophyll-protein complex
pH	-	potential hydrogen
PRR	-	Pathogen recognition receptors
q	-	quantitative
RNA	-	Ribonucleic acid
RNApol	-	RNA polymerase
rpm	-	revolutions per minute

RPMI	-	Roswelli Park Memorial Institute
RSV	-	Respiratory Syncytial Virus
RT	-	Room temperature
RT-PCR	-	Real Time Polymerase Chain Reaction
RAR	-	retinoic A receptor
RXR	-	retinoic X receptor
secs	-	Seconds
SR	-	glucocorticoid resistant
SS	-	glucocorticoid sensitive
Syk	-	Spleen tyrosine kinase
TGFβ	-	Transforming growth factor β
TIMP-1	-	Tissue inhibitor of metalloproteinases – 1
TLRs	-	Toll-like Receptors
Tr1	-	T regulatory cell 1
Treg	-	T regulatory cell
TSDR	-	Treg-specific demethylated region
U	-	unit
U.K.	-	United Kingdom
U.S.A.	-	United States of America
VDBP	-	Vitamin-D Binding Protein

VDR	-	vitamin D receptor
VDRE	-	vitamin D3 response element
µ	-	micro

Table of Contents

List of Tables

List of Figures

1. Introduction

1.1 Immune mechanisms of respiratory health

The ultimate aim of the immune system in the lung is to maintain respiratory health involves the clearance of pathogens from the lung in the absence of excessive inflammation. There are a number of mechanisms in place to prevent inappropriate immune responses to innocuous stimuli such as environmental and commensal stimuli that constantly bombard the lungs. Environmental insults inhaled can include diesel exhaust particles, metals, allergen (House dust mite, cat dander, grass etc.), lipopolysaccharide (LPS), amongst others [1]. As well as the external environmental stimuli there are also a large number of commensal bacterial present in the airways, reports of up to 2000 bacterial genomes detected in just a cm^2 of lung surface [2]. However, in healthy individuals the lungs need to ignore non-harmful external and internal stimuli and maintain respiratory health. When a pathogen or environmental trauma occurs in the lung an immune response is only initiated once the stimuli received is over a certain threshold [1]. However there are some individuals where an inappropriate immune response is initiated to an innocuous antigen, an example of this is asthma, where there is an inappropriate immune response to antigen such as allergens.

When an environmental insult or pathogen enters the lung the components of the immune system need to work in harmony to eliminate the pathogen, and then restore the lung back to homeostasis. The two main sections of the immune system include; the innate cells (immediate response, non-specific) and the adaptive immune system (slower, specific-immune response).

1.1.1 Mechanisms of a healthy lung

In a healthy lung the predominant cell type in the alveolar lumen is alveolar macrophages and they make up around 95% of the cell population [1]. These cells are less efficient at antigen uptake and presentation as they do not express high levels of

costimulatory molecules such as HLA and CD80 or CD86 in high levels. The epithelium cells lining the airways are proposed to modulate alveolar macrophage function. The mechanisms employed by the epithelium prevent an inappropriate immune response in the lung via activation of alveolar macrophages are shown below:

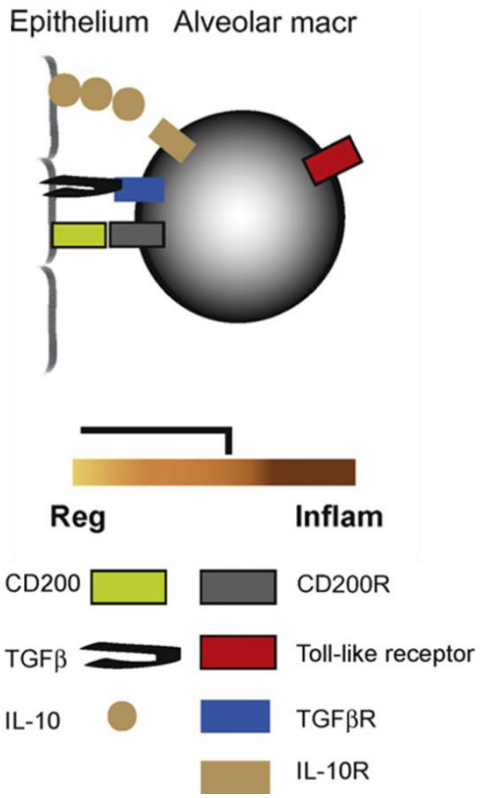


Figure 1-1 Mechanisms of the epithelium preventing activation of alveolar macrophages during lung homeostatsis.

Alveolar macrophages interact with the epithelium through a number of receptors including IL-10R (brown) which binds IL-10, TGFβR (blue) which binds TGFβ and CD200R (blue) which binds CD200, these interactions under homeostatic conditions prevent the activation of alveolar macrophages. Alveloar macrophages also express Toll-like receptors (red) which detect invading pathogens and can initiate an immune response. Taken from [1]

Alveolar macrophages are known to secrete a number of immunomodulatory mediators such as Prostaglandins and Transforming growth Factor β (TGFβ) which are broadly anti-inflammatory [3]. The secreted TGFβ can bind to the TGFβR expressed on the surface of alveolar macrophages and has inhibitory effects on the cell. TGFβ is

pleiotropic and as well as controlling alveolar macrophages also plays a role in wound healing and repair as well as fibroblast remodelling [4]. CD200R is highly expressed by airway macrophages and binds to the ligand CD200 expressed on airway epithelium. Binding of CD200 to CD200R on airway macrophages results in an inhibitory signal being sent to the macrophage [5].

A number of lipids and surfactant (SPD) proteins are also present in the airway. The primary function of these molecules is to decrease the surface tension of the air fluid and keep the lungs open. Goblet cells secrete mucus which contains the glycoprotein Mucin, this acts as a trap for potential invading pathogens and prevents them binding to the epithelium [6]. Mucin-1 and also Surfactant proteins (SPD-A and –D) play an additional role of binding to pattern recognition receptors (discussed later) and prevents ligand binding and activation of these receptors [7-9].

The other 5% of cells that can found in the airway lumen include Dendritic cells (DCs) and T cells (γδ and αβ TCR). The DC predominantly sits under the airway epithelium constantly sampling the airway lumen and in steady-state conditions has been shown to secrete large amounts of IL-10 [10].

When a pathogen or environmental stimuli enter the lung and stimulate the immune receptors and cells over the required threshold there is initiation of an immune response and this begins with the innate immune system.

1.1.2 Innate Immune system

The first line of defence in the lung is the mucosal epithelium, which acts as a mechanical barrier to invading pathogens. On top of the epithelium, there is a thick layer of mucus, produced by goblet cells, this acts as a mechanical trap for invading pathogens. Then due to the pulsation of the cilia present on the epithelial cells the pathogen is expelled. Anti-microbial peptides such as β-Defensins and Cathelicidin are present in the present in the lung, and in particular the mucus layer. Anti-microbial

peptides are predominantly made by the epithelium and innate cells; they are cationic proteins that disrupt the cell membrane and thus kill the pathogen [11,12].

1.1.3 Pattern recognition receptors

The epithelium detects invading pathogens and directs the immune response accordingly through recruitment of further innate and adaptive immune cells. It does this through receptors present called Pattern Recognition Receptors (PRR) which detect Pathogen Associated Molecular Patterns (PAMPs) on the invading pathogen [6]. Examples of PRRs include Toll-like Receptors (TLRs), C-type lectins and Nod-like Receptors (NODs).

1.1.3.1 Toll-like receptors (TLR)

There are a number of TLRs in mice and humans and they recognise common components on pathogens.

TLR family member	Ligands (origin)
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (RSV) Envelope proteins (MMTV)
TLR5	Flagellin (bacteria)

TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds)
TLR8	ssRNA (viruses) Imidazoquinoline (synthetic)
TLR9	CpG DNA (bacteria)
TLR10	Unknown

Table 1-1 TLR family members and their ligands. Taken from [13]

TLRs act as adjuvants for the immune system as upon ligand engagement of TLRs there is an initiation of a signalling cascade which results in upregulation of cytokine production.

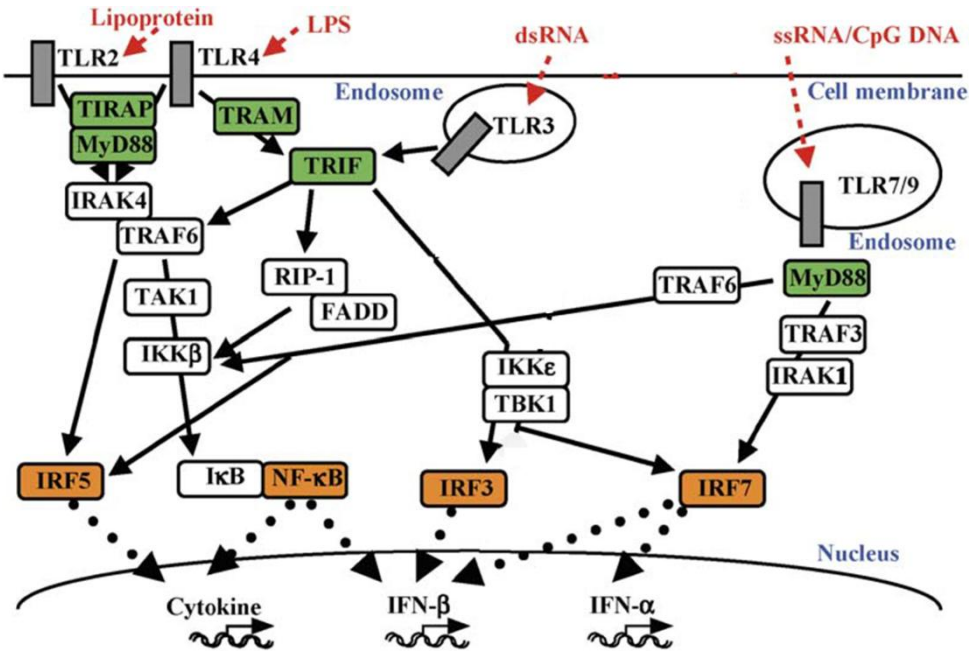


Figure 1-2 Schematic of signalling cascade post TLR ligation.

TLRs can be extracellular such as TLR2 and TLR4 or intracellular such as TLR3 and TLR7/9 found in endosomes. When TLRs bind to their antigen, there is initiation of a signalling cascade which can be either Myeloid differentiation primary response protein 99 (MyD88)-dependant or MyD88-independant. These signalling cascade results in activation of a number of transcription factors such as IRF5 and NFκB which initiate transcription of inflammatory cytokines, as well as IRF7 and IRF3 which activate transcription of Interferon (IFN) genes (IFNβ and IFNα). Taken from [14]

Upon TLR engagement by their respective ligand, the TLR signals through TIR-containing adapters (labelled in green), and the resulting signalling cascade results in the translocation of their respective transcriptions factors (labelled in orange) and upregulation of inflammatory cytokines [14]. Examples of inflammatory cytokines include Interleukin (IL) -6, CXCL8, IL-1β, CCL20, GM-CSF and G-CSF). These cytokines then function by recruiting innate and adaptive immune cells to the lung and also by activating DCs [6,11].

1.1.3.2 C-type lectins

These PRRs are important for recognising fungal pathogens, examples of C-type lectins include Dectin-1 and -2, DC-sign and Mannose receptor [15]. These receptors recognise key components of the fungal cell wall such as Mannan and β-glycan. Ligation of the C-type lectins results in a signalling cascade which results in upregulation of inflammatory cytokines [15]. There is diverse usage of signalling pathways for C-type lectins, as every receptor seems to use a different pathway. Examples of C-type lecting signalling pathways include DC-SIGN, which when bound by ligand results in activation of RAF1, which through an unknown mechanism enhances TLR-mediated production of IL-10 [16]. RAF1 activation in combination with a co-receptor, suggested to be CD4, has also been shown to activate MAPK pathway, which through an unknown mechanism degrades inflammatory cytokine mRNA (such as TNFα and IL-6) [16]. Another C-type lectin, BDCA-2 (CD303) signals through activation of Spleen tyrosine kinase (Syk), which further activate Bruton's typrosine kinase (BTK), which in combination with PLCγ2 activates BLNK. This then leads to enhance Calcium mobilisation in the cell and inhibits TLR-mediated MyD88 signalling [16].

1.1.3.3 Nod-like Receptors (NODs)

Nod-like Receptors (NODs) are intracytoplasmic receptors whose structure consists of a C-terminal leucine-rich repeat domains, a central nucleotide oligomerisation domain and a N-terminal signalling domain [6].

NOD (alternative names)	Ligand or pathogen
NOD1 (CARD4)	γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) (a dipeptide) from Bacillus subtilis, Listeria monocytogenes, entropathogen (Escherichia coli), Shigella Flexneri,

	Pseudomonas aeruginosa, Chlamydia pneumoniae, Campylobacter jejuni and Helicobacter pylori
NOD2 (CARD15)	Muramyl dipeptide (MDP) from Streptococcus pneumonia, Mycobacterium tuberculosis, Listeria monocytogenes, Salmonella typhimurium, Shigella Flexneri and Staphylococcus aureus
NLRP1 (NALP1)	Muramyl dipeptide (MDP)
NLRP3 (NALP3, CIAS1 and Cryopyrin)	Crystals (uric acid, calcium pyrophosphade dehydrate), extracellular ATP, fibrillar amyloid-β peptide, hyaluronan, pollutants (silica and asbestos), bacterial and viral RNA, poly IC, toxins (nigericin andmaitotoxin), UV light, vaccine adjuvant (alum), fungi (Candida albican and Saccharomyces cerevisiae), β-glucan, bacteria (Listeria monocytogenes and Staphylococcus aureus), Viruses (Sendai virus, adenovirus and influenza virus)

Table 1-2 NOD-like receptors and their ligands. Taken from [17]

In general members of the NODs contain three domains. Firstly a caboxy-terminal LRR domain which is responsible for ligand recognition, a central NOD (also known as a NACHT domain) which has ATPase activity, as well as a amino-terminal domain which has protein-protein interaction domains [18]. Ligation of NODs results in an intracellular signalling cascade with upregulation of inflammatory cytokines. For example, ligation of NOD1 and NOD2 by its ligand prevents the proteosomal degradation of NFκB through ubiquitination of the NFκB inhibitor, IKKγ [18]. Additionally NOD1 and 2 have been shown to activate the mitogen-activated protein kinases (MAPK) signalling pathway which results in activation of a number of inflammatory mediators such as JUN amino-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK) and p38 MAPK [18].

1.1.4 Innate Immune cells

The innate cells of the respiratory system include polymorphonuclear leukocytes (granulocytes), neutrophils, eosinophils, basophils, mast cells and also alveolar macrophages.

1.1.4.1 Polymorphonuclear leukocytes

Eosinophils, Basophils and Mast cells contain granules which when activated by either cytokines or receptor (for example FcεRI) binding results in exocytosis of their granular content. The granular content is a number of inflammatory mediators which act in concert to recruit more immune cells; examples of content include Histamine, Prostaglandins and Leukotrienes amongst others [19]. Neutrophils eliminate microbes through phagocytosis and fusion with granules that contain reactive oxygen species and reactive nitrogen species, these free radicals result in efficient killing of the pathogen [20]. As well as this Neutrophils have also been shown to secrete Neutrophil Extracellular Traps (NETs) which contain DNA, histones, neutrophil elastase, myeloperoxidase as well as Cathelicidin. NETs act as traps for extracellular bacteria and immobilise the pathogen making killing more efficient [21].

1.1.4.2 Alveolar Macrophages

As stated before in a healthy lung around 95% of the cells within the airway lumen are alveolar macrophages, in steady-state conditions they produced anti-inflammatory cytokines and receive signals from the epithelium such as CD200 sending inhibitory signals to the cell [1,5]. However in an inflammatory environment, there can be upregulation of TLR receptors on the surface of alveolar macrophages [22]. Subsequently signalling through TLRs can result in inhibition of IL-10R signal transduction and hence release from the inhibitory signals received from the epithelium [23]. Activation of the alveolar macrophages results in increased phagocytosis and

enhanced production of reactive oxygen species and reactive nitrogen species [24]. There is also upregulation of costimulatory molecules allow alveolar macrophages to become more efficient antigen presenting cells for infiltrating T cells [24]. Upon activation alveolar macrophages also increase expression of pro-inflammatory cytokines such as IL-6 and TNFα [24].

1.1.5 Dendritic cells

The sentinels of the immune system are Dendritic Cells (DCs). DCs are constantly sampling the environment, through phagocytosis and macropinocytosis. DCs express a full array of PAMP receptors such as TLRs and NODS, and also express a number of stress receptors such for Danger Associated Molecular Patterns (DAMPs) which also detect cell death or stress (examples of such include uric acid and Adenosine Triphosphate (ATP)) [25]. When a DC either detects cell stress or a pathogen, the DC will mature and migrate to the lymph node to initiate an adaptive immune response. The DC will present peptides from the foreign antigen on MHC, and in the presence of costimulation (CD80 and CD86), this binds to the T Cell Receptor (TCR) and CD28 respectively on the naïve T cell and activates this cell.

There are two classes of DCs, plasmacytoid DCs (pDCs) and myeloid (mDCs). Plasmacytoid DCs were primarily identified as interferon producers, hence were proposed to be important for combating viral infections. Plasmacytoid DCs have also been shown to be important for cross-presentation of exogenous antigens to CD8+ T cells [26]. Additionally pDCs have been shown to be important for tolerance as pDCs have the capacity to prevent autoimmunity through deletion of autoreactive T cells in the liver [27]. mDCs have been further characterised into a number of subsets based on function and phenotype. One such of these subsets include BDCA-3+ (CD141+) mDCs which have been shown to be efficient at cross-presentation as well as enhancing Th1 cells [28]. These BDCA-3+ mDCs have also been shown to be

enhanced in the skin by Vitamin D, and were shown to enhance IL-10 secretion [29]. More recently CD56 expression on human mDCs has been described as cytotoxic mDCs, with potent anti-tumour capability [30].

1.1.6 Adaptive Immune System

There are two classes of T cells CD8+ (cytotoxic) and CD4+ (helper). If antigen is presented in the context of MHC Class I then this activates CD8+ T cells, also known as cytotoxic T cells. These cells function by killing cells expressing peptide and MHC I molecules, and specifically kill the infected cell via secretion of molecules such as Granzymes A and B. They can also synthesize significant quantities of cytokine [31].

1.1.6.1 T helper cell differentiation

CD4+ T cells also known as T Helper (Th) (or T effector) cells were originally described to be either Th1 or Th2 cells as defined by expression of the transcription factors Tbet and GATA3 respectively [32-35]. These cells recognise antigen presented on MHC II molecule, together with costimulation molecules on the surface of antigen presenting cells and this results in production of effector cytokines such as Interferon γ (IFNγ) (for Th1) and IL-4 (for Th2). More recent research has shown that there are more than just these two subtypes of CD4+ cells.

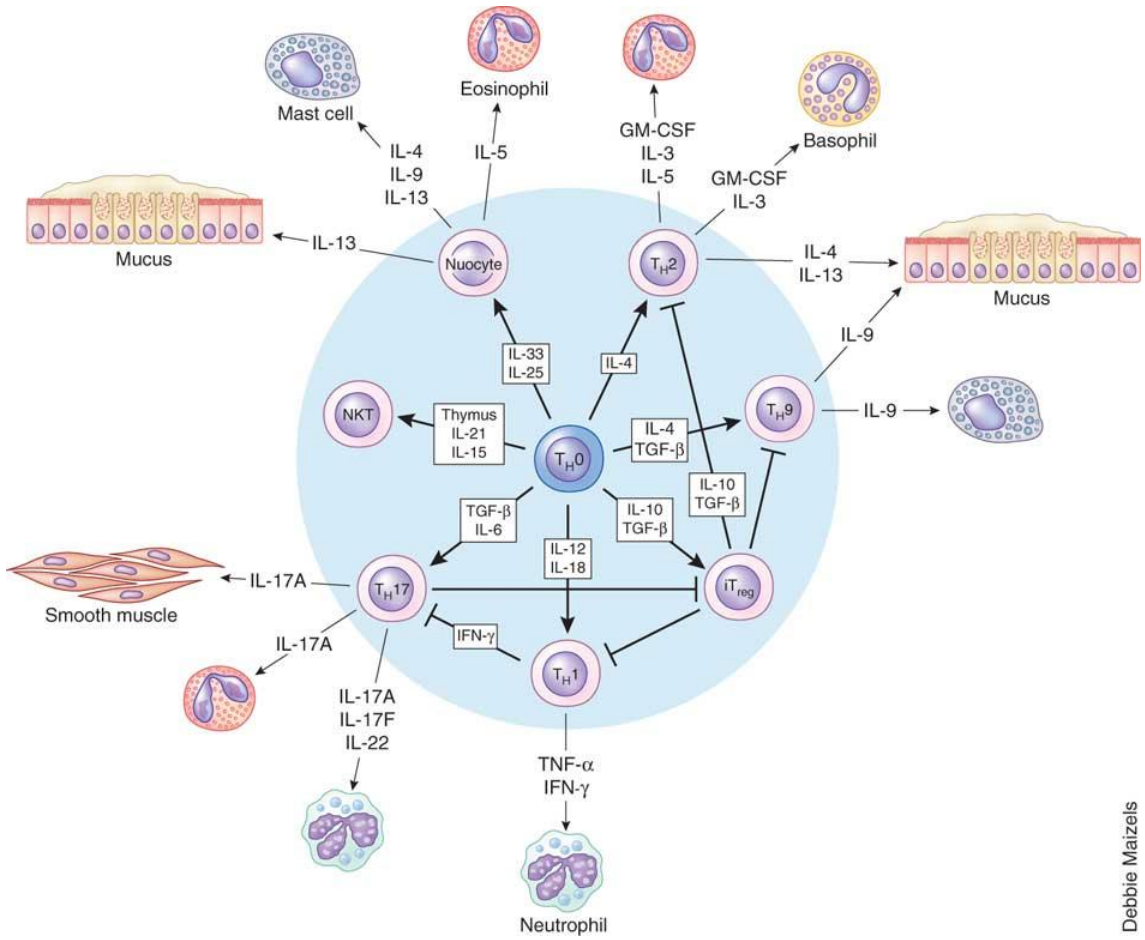


Figure 1-3 Schematic of CD4+ T cell subtypes and effector functions.

A number of CD4+ T cell subsets have been shown to play a role in lungs function. These include Nuocytes which differentiate in the presence of IL-33 and IL-25, produce the cytokines IL-13, IL-4, IL-9 and IL-5 all of which have a role in mucus production and mast cell and eosinophil recruitment. Th2 cells generated in an environment high in IL-4, produce IL-5 and IL-3 and recruit and activate basophils. Th9 cells generated in an environment high in IL-4 and TGFβ, secrete IL-9 and this increases mucus production. Th17 cells generated by an environment high in TGFβ and IL-6, secrete IL-17A, IL-17F and IL-22 and enhance smooth muscle proliferation and recruit neutrophils to the lung. Th1 cells result from an environment high in IL-12, secret IFNγ and enhance neutrophil recruitment to the lungs. iTregs differentiate in the presence of IL-10 and TGFβ, and are important cells for controlling the other CD4+ T cells through a number of inhibitory mechanisms discussed later. Cytokines in the black box indicate what cytokines direct Th0 differentiation into which CD4+ subsets. Taken from [36]

The cytokine milieu plays a role in instructing the naïve CD4+ T cell in what it becomes. For example IL-4 instructs the naïve cell to become Th2 and IFNγ and IL-12 instructs the naïve cell to become Th1, whereas IL-6 and TGFβ instructs the cell to become Th17. There is also a level of plasticity within the CD4+ T cell subsets as the cytokine environment can also change the subset of Th cell [37].

Th1 cells (as defined by the transcription factor T-bet) orchestrate the killing of intracellular pathogens through production of IFNγ, TNFα and Granzymes. Th2 cells (as defined by the transcription factor GATA3) are involved in increasing mucus production and IgE synthesis through IL-4 and IL-13, as well as activating basophils through production of GM-CSF and IL-13, and actions on mast cells and eosinophils through IL-9 and IL-5 respectively.

Th17 cells are defined by the transcription factor RORyt (RORc in humans) and are known to secrete the cytokines IL-17A and IL-22 [38,39]. IL-17A is known to be important for smooth muscle hyperplasia and also for neutrophil activation. Other transcription factors that have been suggested to be important in Th17 development include BATF, IRF4 and STAT3, also the Arylhydrocarbon receptor (AhR) has been implicated as being important for Th17 development [40-44]. What is clear in the lung is that IL-17A and IL-22 is important for maintenance of epithelium and clearance of extracellular pathogens, however too much of these cytokines is associated with poor respiratory health, as discussed later.

Th9 cells either differentiate directly from naïve cells when the cytokine IL-4 and TGFβ are present. It is unclear as of yet whether these are distinct from Th2 cells or are just Th2 cells with a different phenotype and Th2 cells can be skewed towards a Th9 phenotype with the addition of IL-25, or IL-4 and TGFβ [45,46]. The Th9 cells do not seem to express the lineage defining transcription factors (GATA-3, Tbet or RORc) thus they have been defined as a new subset [45]. Th9 cells secrete large amounts of IL-9 which is important for eosinophilia and mucus hyperplasia [47].

Additionally to these CD4+ T cell subsets implicated in asthma pathogenesis there are also Th22 and T follicular helper cells. Th22 cells were originally described in the skin and these cells secret large amounts of IL-22 and TNFα [48]. Th22 cells have been shown to be important for wound healing, tissue remodelling and repair as well as protection against enteropathogenic bacteria and HIV infection; these functions are dependent on the secretion of the cytokine IL-22 [48-50]. T follicular helper cells are defined by expression of the transcription factor Bcl-6, express CXCR5 and secrete IL-21, their main role is to support B cells in germinal centres [51]. T follicular helper cells enhance antigen-specific B cell differentiation and antibody production and class switching [51].

There are also populations of regulatory cells present within the lung which play an important role in ameliorating and controlling inflammation and these are discussed later.

1.1.6.2 Innate Lymphoid Cells

Innate lymphoid cells (ILC), lack the specificity of the adaptive immune system, as there is little antigen receptor editing. Examples of such cells include; Natural Killer (NK) cells, γδ T cells, Lymphoid tissue-inducer (LTi) cells, Nuocytes, ILC17 and ILC22 [52]. Whether these cells are distinct populations or are the same cells going through different stages is still under extensive investigation.

Nuocytes (also called natural helper cells and innate helper cells (Ih2)) are one of the newest ILC, and they secrete large amount of Th2-associated cytokines [53]. These cells do not express the markers CD19 or CD3 and thus are termed non-B non-T cells. They have been associated with airway disease in mouse models of allergic asthma however they are yet to be fully characterised in humans [54].

ILCs such as γδ T cells, ILC17 and ILC22, secrete the cytokines IL-17A and IL-22 which are known to activate neutrophils and epithelium. There is conflicting data of the

role of IL-17A and IL-22 production by these cells with some reports suggesting that they are important for tissue homeostasis and other suggesting they may play a role in inflammation [55-58]. Certainly one study looking at the role of IL-17A production from γδ T cells in the lung, was involved in resolution of allergic airway disease [59]. But research is ongoing in this area, before any conclusions can be drawn.

1.1.6.3 B cells

B cells produce antibodies which play a role in neutralising and opsonising antigen and also in activating the complement cascade. Additionally B cells are antigen presenting cells, and can present MHC Class II restricted antigen to CD4+ T cells.

B cells originate from the bone marrow and after deletion of autoreactive B cells enter the periphery, these transitional B cells are defined in humans by the expression of CD21 and IgD, these cells are antigen naive [60]. A population of transitional B regulatory cells (Bregs) has been identified, these cells secrete large amounts of the anti-inflammatory cytokine IL-10 and are defined as having high cell surface expression of CD24 and CD38 [61].

As discussed previously T follicular helper cells assists activation and antibody production from B cells after encounter with antigen. B cells in the lymph node undergo somatic hypermutation enabling them to become more specific for the antigen. All B cells start by making Immunoglobulin (Ig)M and isotype switching occurs in the presence of cytokine produced from CD4+ T cells. 'B1' B cells are primed in the presence of Th1 cytokines IFNγ and IL-12 and secrete high amounts of IL-10, TNFα and IL-6 [62]. 'B2' B cells are primed in the presence of Th2-associated cytokines IL-4 and IL-13, they secrete large amounts of IL-2, IL-4 and IL-13 [62]. Cytokines originating from CD4+ T cells are known to be important for class switching from Immunoglobulin (Ig)M to IgG, IgA or IgE. IgE is important in the lung as it binds to the FcεRI, the high affinity receptor for IgE present on mast cells and basophils, and when antigen (or

allergen) crosslinks the receptor this leads to degranulation of these cells [36].B cells after antibody isotype switching either become memory B cells (CD38+ B cells) or plasma B cells (CD138+ B cells), which are terminally differentiated B cells [63,64].

All these immune cells of the innate and adaptive immune system work together to successfully fight invading pathogens with limiting inflammation. However there are times when there is a breakdown of respiratory health; and this can lead to diseases such as Chronic Obstructive Pulmonary Disease (COPD) and the focus of this work, Asthma.

1.2 Regulatory T cells (Tregs)

In addition to immune cells (effector cells) initiating and driving immune responses, there are also CD4+ T cells that play an important role in ameliorating inflammation and promoting tolerance. Under normal homeostasis Tregs exist in balance with effector T cells [65].

1.2.1 Discovery of Tregs

It was known since the 1960s, that if the thymus was removed from the female mice then an autoimmune disease in the ovaries developed, however if the thymus was returned before 7-days then autoimmunity was prevented [66]. In the early 1970s, Gershon and colleges were credited with coining the term suppressor T cells. It was believed that suppressor T cells worked via secretion of soluble factors (called I-J) which were encoded in the mouse MHC; functioning in a similar way to B cells. Research into suppressor cells in the 1970-80s focussed on this I-J region, and attempts were made to identify the suppressive secreted protein. When there was no progression in this area of research the area of suppressor T cells became less popular [67,68].

The “modern era” of Tregs emerged from studies by Sakaguchi and others in the mid-1990s, as they described “natural” Tregs (nTregs) produced in the thymus [69]. These cells were characterized by the constitutively high expression of the CD25 antigen, the α-chain of the interleukin (IL)-2 growth factor receptor. CD25 is normally only transiently expressed at moderate levels on effector T cells upon activation. CD25+ regulatory cells were subsequently also identified in human peripheral blood [70-72], cord blood [72,73] and thymus [74]. The suppressive ability of human nTregs as determine by *in vitro* suppression assays, was as efficient as seen in mice, [71,75]. Following the identification of these nTreg the characteristics of these cells were further researched. CD4+CD25+ nTregs were defined as being:

- Anergic – nTregs do not proliferate in response to TCR stimulation *in vitro* [76-78]
- Inhibitory of CD4+ and CD8+ T cell proliferation [79,80]
- Inhibitory of cytokine production from effector T cells [79]
- Unable to produce their own IL-2 and yet dependent upon IL-2 [81]– requirement of IL-2 to be produced by effector T cells; CD25+ nTregs are absent in IL-2 knockout mice [82]
- Preventative of autoimmune diseases [76,83] - this is due to the fact that T cell receptor (TCR) repertoire of the nTregs whilst being diverse has been found to often be specific for ‘self-peptides’, hence important for preventing autoimmunity [84,85]

To investigate whether it was the expression of CD25 that rendered the Tregs suppressive, CD25 expression was investigated on CD4+ effector cells. Upregulation of CD25 by *in vitro* stimulation did not render the CD4+ T cells suppressive [86]. Therefore identifying that the nTregs and T effector cells were distinct T cell populations and additionally that there must be a ‘Treg’ factor upregulated in nTregs to render them suppressive.

1.2.2 Foxp3 the Lineage Defining Transcription Factor

The field of nTregs was further enhanced when the lineage defining transcription factor for nTregs was discovered. It was identified first in mice, and subsequently confirmed in humans, that the lineage-specific transcription factor for nTregs was forkhead box P3 (FoxP3) [87-90]. Foxp3 continual expression in Tregs is required for the development and functionality of the nTreg [91]. FoxP3^{+} nTregs are vitally important for self-tolerance and immune homeostasis, as best highlighted by cases of immune dysregulation polyendocrinopathy, enteropathy X-linked syndrome (IPEX), in which patients lack Tregs due to mutations in the gene *FOXP3*. IPEX is a fatal condition due to widespread autoimmune and inflammatory disease from a very early age unless it is treated aggressively by bone marrow transplantation or profound immune suppression. A similar condition, *scurfy*, was also described in mice [87-89].

Due to the fixation and permeabilisation of cells required to identify Tregs by Foxp3 expression, a lot of research has been performed to identify cell surface antigens that uniquely distinguish Tregs from T effector cells or differentiate between different Treg populations. However these unique cell surface antigens have proven elusive. The best cell surface markers to date to identify Tregs, are low to no expression of CD127 (interleukin seven receptor α) as well as high CD25 [92]. More recently several groups, have tried to further characterise surface markers of Foxp3^{+} Tregs using antigens such as CD45RA or CD49d, however to date there is still no consensus agreement on which markers can be utilised to identify all Tregs without permeabilising the cells to stain for Foxp3 [93,94].

1.2.3 Genetics of Foxp3

The Foxp3 locus is shown in Figure 1-4:

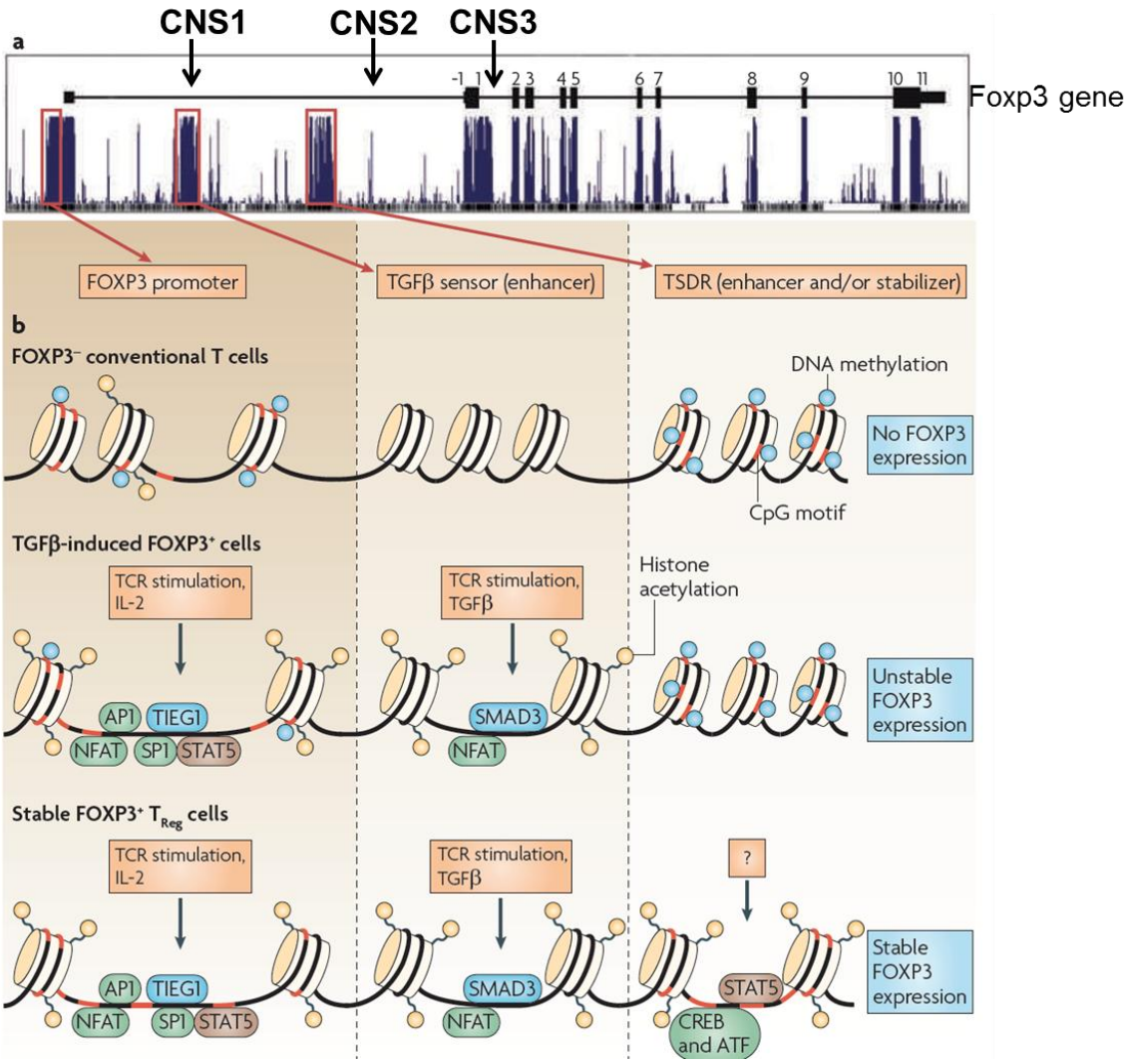


Figure 1-4 Schematic Representation of the Foxp3 Loci

a. Figure showing a schematic of the Foxp3 gene loci CNS1-3 which also contains a Foxp3 promoter, TGFβ sensor enhancer and a TSDR region which all play an important role in Foxp3 gene expression **b.** Chromatin formation at the Foxp3 promoter region in Foxp3- conventional T cells where the gene is 'off', in TGFβ-induced Foxp3+ cells where the promoter and TGFβ the TGFβ enhancer region are 'open' with transcription factors bound and stable Foxp3+ Tregs where all the promoter and enhancer regions of the Foxp3 gene are in the 'open' position and Foxp3 is stably expressed. Taken from [95,96]

As can be seen from figure 1-4 above there are eleven exons in the Foxp3 gene found on the X chromosome, preceded by the promoter region. The promoter region of the Foxp3 gene is a TATA-and CAAT-box-containing promoter, which is activated post TCR-signalling with binding of the transcription factors AP1, NFAT, TIEG1, SP1 and STAT5 [97]. Due to the fact that TCR signalling can initiate Foxp3 expression, there is low level Foxp3 gene induction in T effector cells which is not maintained [97]. As shown in the figure above for Foxp3 to be stably expressed a region within the Foxp3 locus called Treg-specific demethylated region (TSDR) needs to be demethylated [98]. The Foxp3 region also contains three Conserved Non-coding Sequences (CNS), these regions are important for gene induction and maintenance of Foxp3 expression [95]. It has been determined that CNS3 may facilitate opening of the FoxP3 locus making the locus more amenable for transcription. CNS3 has a c-Rel motif, and it is believed to be important for increasing the frequency of Foxp3+ Tregs in the thymus [95]. CNS2 is not required for Foxp3 induction but for maintenance of expression; Foxp3 is known to bind to this element to maintain Foxp3 expression in the Tregs and progeny of dividing cells [95]. CNS1 contains a TGFβ-NFAT response element and is not required for nTreg differentiation; however it is required for iTreg induction [95,99]. When CNS1 KO mice are used it is seen that there is extensive Th2 mediated inflammation in the intestine and the lung [100]. Mucosal sites are known to be important sites for iTreg induction (discussed later).

Although a lot of research has been performed to establish how Foxp3 is expressed it is still relatively unknown how Foxp3 targets its genes as there is no clear Foxp3 target sequence like those seen for other transcription factors, e.g. GATA3 binds to 5'-(A/T)GATA(A/G)-3' [101]. Foxp3 when bound to Runx1 inhibits the expression of IL-17A thus prevents naive CD4+ differentiation into Th17 cells and favours Foxp3+ Treg, conversely RORyt has been shown to inhibit Foxp3 gene expression. Implying that there is a complex interaction where Foxp3+ Tregs negatively regulate Th17 cells, yet

conversely RORγt can inhibit Foxp3+ Tregs generation and thus promote Th17 cell differentiation [102].

Foxp3 and its associated transcription complex was purified from Foxp3 expressing cells and it was found to be in a complex of around 400-800kDa in size [103]. The composition of this complex was suggested to be important for targeting Foxp3 to its target gene as it included a number of sequence-specific transcription factors. It has also been shown that Foxp3 can interact with chromatin modifying enzymes such as histone acetyl transferases (HATs) and histone deacetylases (HDACs) [103]. Thus Foxp3 has the ability to make genes open to transcription via HATs acetylation of histones, making the DNA permissive for transcription. Conversely, Foxp3 can also inhibit transcription with HDACs, which remove the acetyl group and thus the histone and DNA become more packed and thus less available for transcription [104].

1.2.4 Induced Tegs (iTregs)

A number of non-nTregs have been identified to date and these have been collectively termed induced or adaptive Tregs (iTregs) [105].

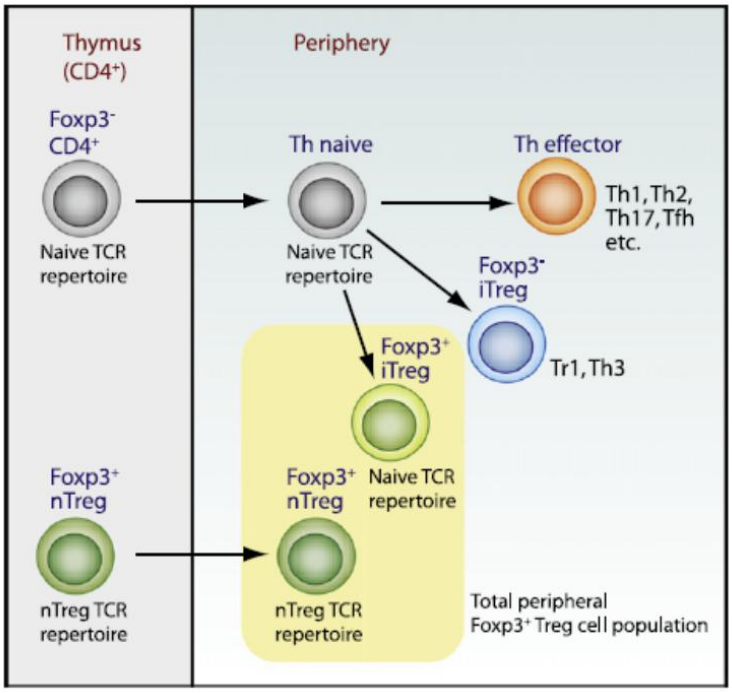


Figure 1-5 Schematic showing the different populations of Tregs.

Foxp3+ Tregs can either originate from the thymus (nTregs) or can be generated from naive Foxp3-CD4+ T cell from the periphery, the combination of these Foxp3+ Tegs form the total peripheral Foxp3+ Treg cell population. Additionally Foxp3- CD4+ Tregs can be generated from naive CD4+ T cells in the periphery, and they can be termed Tr1 or Th3. Taken from [106]

Induced Tregs are generated in the periphery from CD4+Foxp3- cells. iTregs cells may or may not express Foxp3; however they all collectively exhibit regulatory function.

1.2.4.1 Foxp3+ iTregs

Early evidence of conversion of naïve CD4+ T cells into Foxp3+ Tregs was observed when naïve CD4+ T cells were transferred into lymphopenic hosts. It was shown that there was a generation of suppressive Foxp3+ Tregs from the donors cells [107]. In conjunction with this it has been observed in humans that the frequency of Tregs increases with age, and yet conversely the thymus produces fewer cells with age [108].

Together these data provided evidence that there were additional populations of Foxp3+ Tregs that were not nTregs.

Low dose antigen exposure, predominantly at mucosal sites has been demonstrated to be enough to generate a population of iTregs [109,110]. This conversion was enhanced in the presence of TGFβ [109]. TGFβ on its own, was subsequently shown to be capable of generating a population of $Foxp3^+$ iTregs, however the expression of Foxp3 in these cells is unstable [111-113]. However if TGFβ and Retinoic Acid (RA) (which is found in abundance in the gut) are present at the same time this generates a stable $Foxp3^+$ iTreg population [114-116]. This predominantly occurs in the gut, and in mice has been identified to be facilitated by $CD103^+$ DCs, the human equivalent of these DCs is yet to be fully established [117].

More recently evidence suggests that the microbiota at mucosal surfaces can play an important role in iTreg induction. It has been found that human commensals have the ability to drive iTreg induction, as mice colonised with human commensals generated $Foxp3^+$ Tregs with suppressive capacity that genetically resembled iTregs [118,119]. It is believed that these commensal bacteria work by generating an environment which promotes Treg generation in particular through production of TGFβ [119]. The TCR of these Tregs are different to nTregs as they have TCRs specific for components of commensal bacteria [120].

As both nTregs and iTregs express Foxp3, extensive research has been carried out to identify a marker which could distinguish between these two Treg populations. Originally it was believed that Helios an ikaros family member was a marker for nTregs, hence $Foxp3^+$ iTregs could be identified by a lack of Helios [121]. However subsequent work, found that it actually depends on the nature of stimulation *in vitro*, and Helios can be expressed in induced Tregs [122,123]. Helios expression has now been associated as being involved in Th2 differentiation [124], and also may be a marker of CD4+ T cell activation and proliferation [125]. Neuropilin, a receptor which was identified to be involved with neuron axon guidance, has subsequently been shown to be important at

the immune synapse and vital for DC-T cell interaction [126]. Has become a new candidate marker for nTregs, as it is not expressed on Foxp3$^+$ iTregs, however more research will need to be performed to identify how good a marker this is as Neuropilin is upregulated on all Tregs at sites of inflammation [127,128]. In addition this work was carried out exclusively in mice and a human study has shown that Neuropilin is not expressed on any Foxp3+ Treg population, and is actually suggested to be a T cell activation marker [129]. So to date, a clear marker that can distinguish between these two Foxp3$^+$ Treg populations has yet to be found.

1.2.4.2 IL-10+ Tregs (Tr1)

There is also a population of iTregs that do not express Foxp3 but instead secrete a large amount of the anti-inflammatory cytokine IL-10, called T regulatory 1 cells (Tr1). One of the earliest identifications of IL-10+ Tregs was observed in severe combined immunodeficiency (SCID) patients, where CD4+ T cells secreting large amounts of IL-10 were important for successful transplantation of hematopoietic stem cells [130]. These cells were subsequently recreated *in vitro,* when OVA-specific CD4+ T cells were cultured in the presence of IL-10, this generated CD4+ T cells that secreted large amounts of IL-10 and lacked proliferative potential [131]. When the IL-10+CD4+ T cells were transferred to a model of colitis, it was found that they controlled the disease. These cells were coined Tr1 cells [131]. Tr1 cells are generated *in vivo* by immature DCs secreting large amounts of IL-10 in the presence of antigen [10,132]. Conversion of Th1 and Th2 cells to Tr1 cells can be induced in mouse models of autoimmunity through repeated exposure to antigen [133]. The generation of these antigen-specific Tr1 cells is dependent on strong signal strength, peptides with less avidity for the TCR are less likely to become Tr1 cells [134]. There is also evidence that T cells when stimulated in the presence of CD46 (the complement regulator protein), under homeostatic conditions, give rise to Tr1 cells [135].

1.2.5 Mechanisms of Treg Function

There has been much research trying to identify the numerous mechanisms by which Tregs control immune responses, and these are listed below.

Mechanism	Function	Reference
CD39/CD73	CD39 and CD73 are cell surface markers involved in the breakdown of extracellular ATP (performed by CD39) to AMP which is then converted to the immunoregulatory molecule Adenosine (performed by CD73). Adenosine then binds to A2A receptors on T effector cells and inhibits their function. Also, CD39 expression on Tregs has been proposed to play an important role in controlling Th17 cells.	[136-140]
Cytotoxic T-lymphocyte Antigen 4 (CTLA-4)	Originally it was believed that CTLA-4 functions through competitive inhibition with CD28 for binding to CD80 and CD86, thus preventing T effector cell activation. Also there is evidence that CTLA-4 delivers a cell-intrinsic negative-signal which inhibits T-cell activation. However, more recent data has identified that CTLA-4 can capture CD80 and CD86 from opposing T cells by a process of trans-endocytosis and targeted these molecules for degradation by the lysosome. Certainly, a genetic defect in CTLA-4 leads to a widespread autoimmune disease.	[141-146]
Galectin-10	Unknown function and regulation. However highly expressed in human Tregs, and knockdown by siRNA prevented Treg suppression of effector T cells.	[147]

Granzyme/ Perforin	Although originally thought to be only expressed in CD8+ T cells, it has now been identified that CD4+ Tregs express cytotoxic granules including Granzyme B and Perforin. When exocytosed these cause apoptosis of target cells.	[148-150]
IL-2 consumption	Tregs have high expression of CD25 and also the inability to make their own IL-2. Thus one of the main mechanisms by which Tregs inhibit T effector cells is through consumption of IL-2 and thus generate cytokine-deprivation induced apoptosis in the T effector cells.	[151]
IL-10	An anti-inflammatory cytokine produced by a wide range of immune cells, including DCs, macrophages and Tregs. IL-10 has many functions these include inhibiting T effector proliferation and also rendering DC more tolerogenic. Also suppresses IgE production from B cells and also through some mechanism yet to be identified mast cells, eosinophils and basophils.	[152-154]
IL-35	An immunosuppressive cytokine a member of the IL-12 family, that is made up of two subunits *Ebi3 (*Epstein-Barr-virus-induced gene 3) and *IL12a (*interleukin 12a), and is highly upregulated in Tregs. It is believed to have an important role for Tregs in suppressing T effector proliferation.	[152,155]
Lymphocyte Activating	LAG-3 is an MHC-class-II-binding CD4 homolog and is expressed by Tregs. LAG-3 is	[156]

Gene 3 (LAG3)	proposed to inhibit T effector cell proliferation	
Programmed death 1 (PD-1)	PD-1 engagement with its ligand PDL-1 generates an inhibitory signal, resulting in inhibition of proliferation and cytokine secretion in the PDL-1 expressing cell.	[157,158]
Transforming Growth Factor β (TGFβ)	TGFβ is important in development and maintenance of Tregs, with TGFβ-specific binding site present in the promoter region of Foxp3. Also TGFβ has been shown to be expressed by activated Tregs, and inhibits effector T cells in the gut and in maintaining tolerance. Also suppresses IgE production from B cells and also through some mechanism yet to be identified mast cells, esoinophils and basophils.	[95,154,159,160]

Table 1-3 Mechanisms of action of regulatory T cells

1.3 Asthma

Asthma is considered to be one of the most common chronic diseases with an estimated 300 million people with the disease worldwide, and an estimated 5000 deaths annually from the disease alone in the U.S.A. [161,162]. It is estimated that over 15% of the U.K. population are asthmatic (5.4 million people), and the NHS spends one billion pounds a year to treat and care for people with asthma (Asthma U.K., www.asthma.org.uk accessed January 2013) [161].

The main characteristic of asthma is episodic wheeze/cough with obstructive lung function, as determined by Forced Expiratory Volume (FEV_1) to Forced Vital Capacity (FVC) ratio. Common triggers that exacerbate asthmatic disease include allergens, pollution, cold air, exercise, virus infection and smoking. Asthma significantly impacts the quality of life of patients by day-to-day symptoms of wheeze, cough and breathlessness limiting their quality of life. Asthma exacerbations often require hospitalisation and can be life-threatening.

1.3.1 Immune Mechanisms of Asthma

Asthma is a chronic inflammatory airway disease characterised by airway hyperresponsiveness, reversible bronchoconstriction, mucus hyperplasia and tissue remodelling. Asthma was historically thought to be a Th2-mediated disease with a predominantly allergic response to an innocuous antigen with high levels of Th2 cytokines within the airways including IL-4, IL-5, and IL-13. These mediators stimulate IgE-mediated mast cell degranulation (leading to a release of bronchoconstrictors), eosinophil infiltration and activation, mucus hyperplasia and airway remodelling [163]. This chronic inflammation, due to the inflammatory milieu leads to disease specific airway remodelling, where there is progressive loss of function and eventual permanent irreversible airway obstruction. Factors which contribute to airway remodelling, including airway muscle cell hyperplasia, and an imbalance of Matrix

metalloproteinases (MMPs) and Tissue inhibitor of metalloproteinases (TIMP) leading to deposition of extracellular matrix [164].

However asthma is in fact heterogenous and can present in many different phenotypes with different distinctive features.

Asthma phenotype	**Description**
Atopic (allergic) asthma	○ Reactivity to one or more known allergens (determined by skin prick test) ○ Higher than normal IgE levels ○ Specific IgE to allergen ○ Often associated with a history of eczema and/or hayfever
Non-allergic asthma	○ No known allergy ○ Normal serum IgE ○ No reactivity to allergen
Mild asthma	○ Occasional symptoms, but well controlled by therapy (low-dose glucocorticoids and/ or short-acting β_2 agonists)
Stable asthma	○ Well-controlled symptoms ○ No lung function variability or asthma exacerbations
Severe asthma	○ Poor control of symptoms despite using maximal therapy (high-dose glucocorticoids and long-acting β_2 agonists, and often other therapies) ○ Associated with Th17-mediated disease
Asthma exacerbations	○ Mild or severe asthma with sudden worsening

	of symptoms, often requiring medical consultation or hospitalization and possible oral glucocorticoid intervention
Neutrophilic asthma	○ Increased number of neutrophils in bronchoalveolar lavage or sputum samples (can be allergic or non-allergic asthma)
High T_h2-associated asthma	○ Th2 cell-associated gene expression is typical (for example, expresses *IL13* and IL-13-inducible genes) ○ Responds well to glucocorticoids ○ Prone to asthma exacerbations
Low T_h2-associated asthma	○ A mixed phenotype of asthma ○ Less prominent Th2 type cytokine responses ○ More refractory to glucocorticoid treatment than patients with high Th2-associated

Table 1-4 Asthma phenotypes.

Taken from [165,166].

Thus asthma is no longer seen as just a predominantly Th2-mediated disease and as such each phenotype has clearly different clinical and immunological features [166,167]. It has become important to phenotype the asthma presented and then specifically administer a therapeutic that targets the specific asthmatic disease. An example of this Lebrikizumab, anti-IL-13 monoclonal therapy, was particularly efficacious in asthmatics that had high serum levels of periostatin (an IL-13 responsive gene) prior to treatment [168].

1.3.2 The role of Tregs in Asthmatic Disease

In mouse models of allergic asthma it has been shown that Foxp3+ Tregs are able to alleviate disease [148,169,170]. Conversely asthmatic disease worsens in these models when Foxp3+ Tregs are depleted [169]. In humans in adult and paediatric cohorts it has been shown that asthmatics have lower Foxp3 expression as well as less suppressive function of their Foxp3+ as compared to healthy controls [171-174]. Certainly environmental exposure to pollution and early life virus infection, known risk factors for asthma development can prevent Foxp3+ gene expression and also inhibits the suppressive function of Foxp3+ Tregs [175-177]. This could be a result of there being a high Th2 environment in asthma, as TGFβ-driven iTreg generation in culture has shown to be inhibited by the presence of IL-4 [178,179].

Immunotherapy is the process of allergen desensitisation, and it involves the subcutaneous injection of increasing doses of the specific allergen the patient is sensitive to. It is a specific treatment for allergic disorders in particular allergic asthma. The mechanisms of action include skewing the immune response to the specific allergen from Th-2 to Th-1 and also induction of Tr1 cells [180,181]. Tr1 cells are vital in immunotherapy as the IL-10 produced by these cells is essential for induction of tolerance to the allergens [181,182]. As well as the role of Tr1 cells in immunotherapy in mouse models of allergic asthma, they have been shown to be important for preventing allergy induced airway hyperresponsiveness [10].

There is clearly a dysfunction of Tregs in allergic and asthmatic disease, Tregs represent an exciting therapy as they can specifically target allergic disease as seen with immunotherapy. Therefore investigating ways to enhance Treg numbers and activity represents an active and exciting area of research in targeting asthma, and modulation of these cells by drugs such as vitamin D holds much potential for combating asthma [153].

1.3.3 Asthma Therapies

1.3.3.1 Glucocorticosteroids

The current cornerstone treatment for asthma is glucocorticosteroids (steroids) Steroids are believed to work via induction of anti-inflammatory genes and turning off of pro-inflammatory genes [183,184].

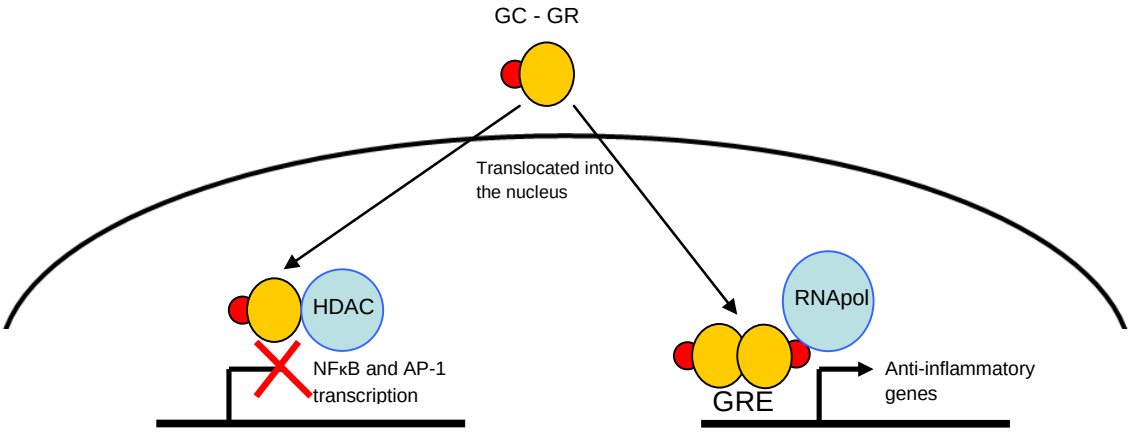


Figure 1-6 A Schematic diagram representing two known mechanisms of action for glucocorticoids.

Glucocorticoids (GC) enter the cell and bind to the glucocorticoid receptor (GR) in the cytoplasm and this binding result in the translocation of this complex into the nucleus. The GC-GR complex can either bind to HDAC and inhibit NFκB and AP-1 transcription or bind to a Glucocorticoid response element (GRE) element in combination with RNA polymerase (RNApol) and initiate transcription of anti-inflammatory genes.

Glucocorticoids enter the cell and diffuse in the cytoplasm where they interact with the Glucocorticoid Receptor (GR). GR is a ligand activated transcription factor, which when bound to GC is translocated into the nucleus. Once in the nucleus GR can either bind to HDAC (an enzyme that acetylates DNA and hence closes the gene for transcription) and turns off genes such as NFκB and AP-1 which are important transcription factors for the inflammatory cytokines [184]. AP-1 and NFκB are important activators of

inflammatory genes, hence glucocortioids turn off inflammatory genes like IL-5 and IL-13 and decrease inflammation via this pathway [183,185]. Alternatively the GR can bind to anti-inflammatory genes via binding as a homodimer to Glucocorticoid Response Elements (GRE) promoters upstream of the anti-inflammatory genes. RNA polymerase is recruited and transcription of anti-inflammatory genes is switched on [183,184]. One of the key anti-inflammatory genes upregulated by steroids is IL-10, and it is believed that the mechanisms for this is GR binding to the GRE in the promoter region of the IL-10 gene [185-187]. Steroids have also been shown in asthmatic patients to upregulate Foxp3+ gene expression [188]. Together steroids induce a regulatory environment through decreasing pro-inflammatory cytokine production and induction of anti-inflammatory cytokines and increase in Tregs.

Steroids work in the majority of patients particularly moderate to mild asthmatics, these patients are known as steroid sensitive (SS) asthmatics. However, there is a proportion of patients that fail to show an improvement in lung function after treatment with a dose of oral steroids and assessment of compliance [189]; these patients pose a serious clinical and a financial burden on the health care system, and are known as steroid insensitive or resistant (SR). There is still an unmet clinical burden in asthma and particularly at the severe end of the asthma spectrum, thus other therapies have been/are being developed for use in asthma.

1.3.3.2 Other Asthma Therapies

The side effects to prolonged and high dose steroids therapy are many, and these include effects on the:

- Skin (acne, delayed wound healing)
- Skeleton (osteoporosis, bone necrosis)
- Eye (glaucoma, cataract)

- Central Nervous System (disturbances in mood, behaviour, memory and cognitive function), Electrolytes (Cushings syndrome, growth retardation)
- Cardiovascular system (hypertension, thrombosis)
- There is increased risk of infection (examples of which include Candida spp.) [190].

Additionally to this there is the additional complication that steroids do not work in all phenotypes of asthma, particularly Th2-low asthma [191]. Therefore a number of additional therapies are used and also being developed to reduce the steroid burden in asthma.

Treatment	Description	References
β2 agonists	○ Widely used therapies that reduce contraction of airway smooth muscle and result in bronchodilation ○ Can be short or long acting	[192]
Leukotriene receptor antagonists	○ Prevent cysteinyl leukotrienes from binding the CysLT1 receptor and inducing airway smooth muscle contraction, mucus secretion and airway inflammation ○ For example, montelukast	[193,194]
Therapies against Th2 type cytokines	○ Monoclonal antibodies and soluble receptors against IL-4, IL-13 and IL-5 ○ Mechanism: reduce circulating levels of Th2 type cytokines	[168,195]
IgE-specific antibodies	○ Monoclonal antibodies (for example, omalizumab)	[196]

	○ Reduce levels of circulating and cell-bound IgE	
Therapies against TNF	○ Soluble TNF receptors or TNF-specific antibodies ○ Reduce levels of circulating TNF ○ Proposed for use in severe asthma	[197,198]
IFNs	○ IFNα had a clinical benefit in steroid-resistant asthma	[199]
Theophyllines	○ Therapy for severe/steroid resistant asthma ○ Mechanisms of action: Adenosine receptor antagonism, increase in Histone deacetylase activity (HDAC)	[200]

Table 1-5 Current and proposed therapies in asthma.

Taken from [165].

The only currently approved biological therapy for asthma is Omalizumab; it is a humanised murine monoclonal antibody which is specific to the FcεR1-binding domain of IgE. It has been shown to reduce serum levels of IgE as well as preventing the allergic asthma exacerbations [196,201]. There are a number of clinical trials that have recently concluded that specifically target Th-2 cytokines; in particular anti-IL-13 with Lebrikizumab and anti-IL-5 with Mepolizumab [168,195]. Lebrikizumab was particularly efficacious in asthmatics that had high serum levels of periostatin prior to treatment [168]. Mepolizumab has been targeted at asthmatics that have severe eosinophilic asthma, and it has just recently been found to reduce asthma exacerbation and hence hospital admissions [195]. Also there have been clinical trials investigating Pitrakinra, a competitive inhibitor of the IL-4Rα receptor which is a shared receptor chain for both IL-4 and IL-13 receptors. This trial was specifically targeted to allergic-asthmatics, it was

observed that there were fewer asthma-related adverse events as compared to the placebo control group [202].

What these data collectively indicate is that asthma therapy is becoming more individual, and targeting specific phenotypes of asthma may be more effective than a non-specific anti-inflammatory drug.

1.3.4 Asthma Development

Although it is not clear what causes a breakdown in respiratory health and initiation of asthma, it is clear that asthma is a complex disease with genetic and environmental associations [203].

1.3.4.1 Genetic associations

Below is a table of some of the gene polymorphisms associated with asthma.

Gene	Function (chromosome position)	Reference
ADAM33	Metalloproteinase (20p13)	[204]
ADRB2	B2—adrenoreceptor; involved in broncial smooth-muscle relaxation (5q31-32)	[205,206]
CD14	Co-receptor with TLR4 in recognition of LPS (15q31)	[207]
DENND1B	Gene expressed by dendritic cells and NK cells and believed to interact with tumour necrosis factor α (TNFα) (1q31)	[208]
FcERIB	High-affinity receptor for IgE (11q13)	[209,210]
GSTP1	glutathione S‑transferase T1; protects cells from reactive oxygen species (22q11)	[211-213]
HLA-DRB1	MHC molecule s (6p21)	[214-218]

HLA-DQB1 *HLA-DPB1*		
IL4Rα	Receptor for IL-4 and IL-13 (16p11)	[219,220]
IL4	Th2-cytokine (5q31)	[221]
IL10	Immunomodulatory cytokine (1q31-32)	[222-226]
IL13	Th2-cytokine (5q31)	[214,220]
IL1RL1	The receptor for the Th2-cytokine IL-33 (2q12)	[215,227]
LTA	Inflammatory cytokine (also known as TNFβ) (6p21)	[209]
ORMDL3	Gene expressed in epithelial cells involved in metalloproteinase and chemokine production (17q12-21)	[227-230]
PTGDR	Prostaglandin DR receptor gene (14q22)	[231]
PDE4D	Phosphodiesterase 4D a cAMP-specific gene (5q12)	[232]
STAT6	Downstream of IL-4 and IL-13 signalling (12q13)	[233,234]
TNF	Inflammatory cytokine (6p21)	[209,235]

Table 1-6 Polymorphisms in genes associated with asthma

Although not an exclusive list it includes genes implicated in asthma susceptibility in over 10 publications according to the following reference [236] and recent genetic associations since publication of that review.

Predominantly these genetic associations occur in genes either involved in immunity in particular Th2 cells and also with maintenance of respiratory health. However, all these genetic associations seem only to be present in certain ethnic populations, thus suggesting that they only increase the susceptibility to asthma [229,237,238]. A number of twin studies have shown there is up to 68% heritability of asthma [239-241], however there is a clear environmental role in asthma development, as these genes only render the individual susceptible to asthma development. The environmental

factors that are also associated with asthma development include vitamin status, infection history, and pollution exposure.

1.3.4.2 Hygiene Hypothesis

The two main hypothesis that still exist around the development of immune mediated diseases including asthma are the 'hygiene hypothesis' and the 'sunshine hypothesis'. Vitamin D will be discussed in more detail in the next section

The hygiene hypothesis was first proposed by Strachan [242]. It proposed that the lack of exposure to microbes in early life, due to the westernised lifestyle of improved sanitation, smaller family size, and the introduction of vaccination and antibiotic resulted in fewer infections in early life. The implication was that the immune response was not 'educated' and therefore develops inappropriate Th2-associated immune responses to innocuous antigens, as in the development of asthma and atopy [165,243]. Subsequently a number of associations were reported between infection history and asthma development. Individuals that had early life exposure to farms, had increased risk of infections of Hepatitis A and *Toxoplasma gondii*, had reduce incidences of asthma and atopy [243-249]. However there were also some studies that countered these earlier findings, as some reports stated that children who grow up on farms had increased incidence of atopy [250], with some data suggesting early life exposure to Helminths was not protective for asthma development [251,252].

However this theory proposed by Strachan, has been suggested to be an oversimplification hence the hygiene hypothesis was modified. This modified hygiene hypothesis proposed that the microbiome of the individual particularly during early had an important role in the education of the immune response particularly as the microbiome is important for development of mucosal tolerance [242,253,254]. It is believed that the neonatal immune system is primed for a more Th-2 associated response, and that there is a requirement for commensals to instruct the immune

system towards a more Th1 and Treg phenotype [255,256]. As in mice reproduced in Germ free conditions have an immune system have a less mature immune system more prone to Th2-asscoiated cytokine production, the additional of communal bacterial products can correct this defect [257]. Additionally there is a report that shows that early life exposure of antibiotics reduces the number of bacteria species in the gut and hence leads to increased incidence of asthma [258]. A recent study, investigating the microbiome in lungs of asthmatics and healthy controls, found that asthmatic lungs contained much fewer species of bacteria. Also it was determined that asthmatics had much higher frequencies of pathogenic bacteria such as *Proteobacteria* and *Haemophilus spp*, whereas the healthy lungs contained a wider variety of commensal bacteria [2].

So clearly the microbiome gut as well as the lung is important for asthma development, as well as educating the immune systems and clearly plays a role in asthma development. However there are also confounding factors for the hygiene hypothesis including Vitamin D sufficiency, diet, socioeconomic status, recurrent viral infections and genetics associated.

1.3.4.3 Pollution

Pollution has long been known to be trigger for asthma exacerbations and has been strongly correlated with hospital admissions [259,260]. The composition of pollutant matter has been assessed and it has been determined that there are many constituents to it including: diesel exhaust particles, heavy metals (such as copper and Zinc), LPS, tyre rubber, and also allergens [261,262]. The presentation of allergens together with the other inflammatory milieu associated with pollutant particulate matter may alter the immune response to the allergen and facilitate atopy and asthma development [261]. Whether early life exposure to the high pollution levels, preludes asthma development is currently being assessed at Kings College London. A

longitudinal study is currently being performed to investigate the implication of the introduction of the low emission zone in London [262,263].

1.3.4.4 Infectious history

Additionally, early life upper respiratory viral infections such as Respiratory Syncytial Virus (RSV) and Rhinovirus that lead to bronchiolitis and/or hospital admission, predisposes to wheeze and asthma development in children and adolescence [264-266]. It has been proposed that RSV infection may be one of the biggest independent risk factors for asthma development [266]. In conjunction with this the time of year you were born has an impact on virus infection and asthma development. Children born 4 months prior to 'winter virus' peak as compared to children born earlier, had 29% increased odds of developing asthma [267]. The mechanism behind how RSV predisposes to asthma has been investigated in a mouse model of allergic asthma, where it was shown that RSV promotes a Th2-environment and also may render Tregs less suppressive [177]. So certainly the hygiene hypothesis and asthma development seems to apply for extracellular pathogens, however there is increasing evidence that intracellular pathogens such as virus actually may predispose to asthma development.

1.4 Vitamin D

The active form of vitamin D is a secosteroid hormone. The majority of Vitamin D is generated in the skin from 7-dehydrocholesterol following exposure to UVB rays from sunlight. It can also originate from diet via fortified dairy products as in the U.S.A., meat, eggs and oily fish [268-270]. The biosynthesis pathway of Vitamin D is shown below:

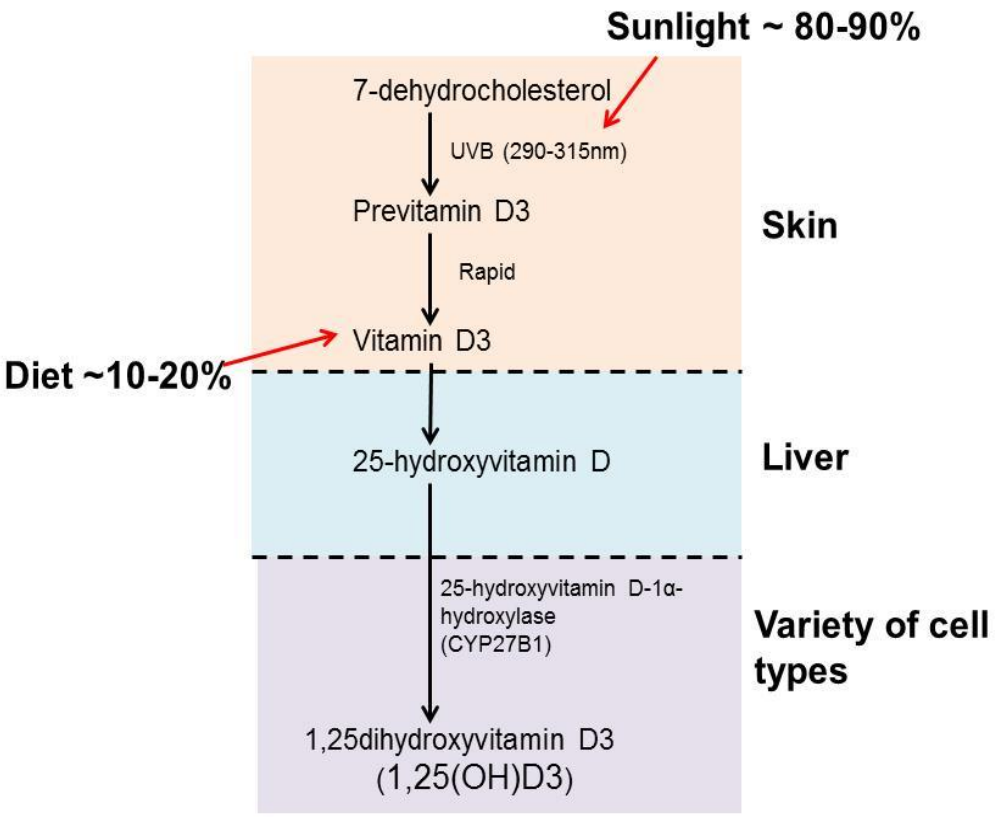


Figure 1-7 Vitamin D metabolic pathway

80-90% of vitamin D is synthesised from 7-dehydrocholesterol in the skin, made into PreVitaminD3 catalysed by UVB light from sunlight. This is rapidly made into Vitamin D3, which can also be obtained from the diet. Vitamin D3 is then made into 25-hydroxyvitamin D in the liver, and then this is subsequently synthesised to the active form of vitamin D 1,25dihydroxyvitaminD3 in a variety of cell types.

Sunlight is absorbed by the epidermal and dermal cells; UVB light splits the B ring of 7-dehydrocholesterol and leads to the production of previtamin D3 [268]. Pre-vitamin D3 is then rapidly converted to Vitamin D3, which leaves the skin and enters the liver. In the liver it is converted to 25-hydroxyvitamin D by cytochrome p450 enzymes (25-hydroxylases). 25-hydroxyvitamin D (25(OH)D) a circulating metabolite is absorbed mainly by kidney cells. More recent evidence has shown that as well kidney cells, a number of other cell types including epithelial cells, macrophages, T lymphocytes and dendritic cells (DC), convert 25(OH)D to the active form 1,25 dihydroxyvitamin D3

($1,25(OH)_2D3$) utilising the mitochondrial enzyme 25-hydroxyvitamin D-1α-hydroxylase [271-274].

When $1,25(OH)_2D3$ enters the cell it binds to the vitamin D receptor (VDR) which is a member of the superfamily of nuclear hormone receptors [275]. Binding of 1,25(OH)D3 leads to a conformational change in the VDR and enables binding to the retinoic X receptor (RXR) and formation of a heterodimer [276]. RXR is a shared nuclear hormone receptor as it can also bind as a homodimer or as a heterodimer with retinoic A receptor (RAR) to the active form of vitamin A, retinoic acid. After heterodimer formation it is translocated to the nucleus where it can exert three different effects: it can bind to a vitamin D3 response element (VDRE) in the promoter region of target genes and turn on genes including genes involved in bone remodelling and bone matrix protein formation, like osteocalcin and osteopontin; it can bind to what is termed a 'negative' VDRE and prevent gene transcription; or it can bind to transcription factors present in the nucleus and prevent them from binding to target genes. These last two effects tend to result in a broad anti-inflammatory effect which is discussed later [276,277].

1.4.1 Vitamin D insufficiency

Vitamin D status is measured by assessing serum levels of 25(OH)D, and it is currently an area of active discussion as to what levels constitute sufficiency and deficiency. There is a lack of agreement within the field, but the broadly accepted level can be seen below:

Vitamin D status	Concentration of 25(OH)D within blood:
Vitamin D deficiency	< 50nmol/L
Vitamin D insufficiency	50-75nmol/L
Vitamin D sufficient	>75nmol/L

Table 1-7 Table showing the definition of vitamin D deficiency and insufficiency [269,278]

Due to the fact that we get the majority of our Vitamin D from a sunlight catalysed reaction, it is not that surprising that during the Winter and Spring months ~90% of the U.K. population is vitamin D insufficient, that only decreases to ~60% in the summer [279]. To achieve vitamin D sufficiency it has been shown during the winter months in Ireland the average person is required to have a daily intake of 9 µg per day of Vitamin D3 [270]. Vitamin D insufficiency has a genetic component leading to susceptibility to low vitamin D status with SNPs being identified in genes associated with cholesterol synthesis, hydroxylation and also vitamin D transport [280]. However the majority of vitamin D insufficiency in Western society has been suggested to occur due to the sedentary lifestyle where there is reduced exposure to sunlight due to sunshine avoidance and working indoors during daylight hours. However there are additional factors which are known to play a role in vitamin D sufficiency including skin colour and obesity. In the U.K. there is no vitamin D supplementation of food as seen in the U.S.A., but even this supplementation programme is not optimum as there is still huge insufficiency in the U.S.A. [268,270].

Vitamin D deficiency results in an increased risk of bone conditions and is strongly linked with rickets and osteomalacia. However, there is also an increased health risk by being vitamin D insufficient and it has been linked to a number of non-skeletal diseases including heart disease [281], breast, prostate and colon cancer incidence and survival [282-286], type 2 diabetes [287], and autoimmune disease such as type 1 diabetes and

multiple sclerosis [288-290]. Certainly there are also SNPs in the VDR gene that is associated with development of immune-mediated diseases such as type-1 diabetes and asthma [291-293]. It has been shown that some Multiple Sclerosis patients have a mutation in Cyp27B1 which means they are less efficient at generating the $1,25(OH)_2D3$ [294,295].

1.4.2 Vitamin D and Respiratory Health

There is also a strong positive association with Vitamin D sufficiency and respiratory health [296]; it has been shown that insufficiency in vitamin D is associated with increased incidences of Tuberculosis [297,298], infections like Respiratory Syncytial Virus (RSV) and Influenza [299,300], Chronic Obstructive Pulmonary Disease (COPD) [301] and Asthma.

1.4.2.1 Vitamin D and Asthma

There is increasing evidence showing that Vitamin D insufficiency correlates with asthma development and severity [278,302]. Certainly low Vitamin D status during pregnancy increases the incidence of wheeze, a surrogate marker used at 3 and 5-years of age before asthma can be readily diagnosed [303,304]. Children and adults with low vitamin D status are most likely to have severe asthma and are less likely to respond to asthma treatment [305-307].

Components of the Vitamin D metabolism and signalling axis have also been associated with asthma. There are a number of SNPs in the VDR gene that has been associated with increased risk of developing asthma [293,308,309]. Another component of the Vitamin D signalling axis associated with asthma is Vitamin-D binding protein (VDBP), VDBP is a protein found in the serum and tissues that has high affinity for 25(OH)D and $1,25(OH)_2D3$ hence sequesters Vitamin D away from target

cells [310]. BAL levels of VDBP were shown to inversely correlate with asthma severity [311].

Collectively this data is driving a research effort that proposes vitamin D supplementation to promote respiratory health. It may in fact be a cheap and efficacious way to help asthma, particularly those at the severe end of the asthma spectrum.

1.4.2.2 Vitamin D and Immune Mechanisms of Respiratory Health

Evidence that the active form of vitamin D is important within the lung is further supported by the fact that respiratory epithelial cells and immune cells that can enter the lung environment have been shown to express the enzyme 25-hydroxyvitamin D-1α-hydroxylase, and hence can generate the active form of vitamin D, 1,25$(OH)_2$D3 [271-274]. This suggests that there is a role for local synthesis in lung environment and hence may play an important regulatory role. It is believed that vitamin D maintains respiratory health via modulating the immune system as shown in the figure below:

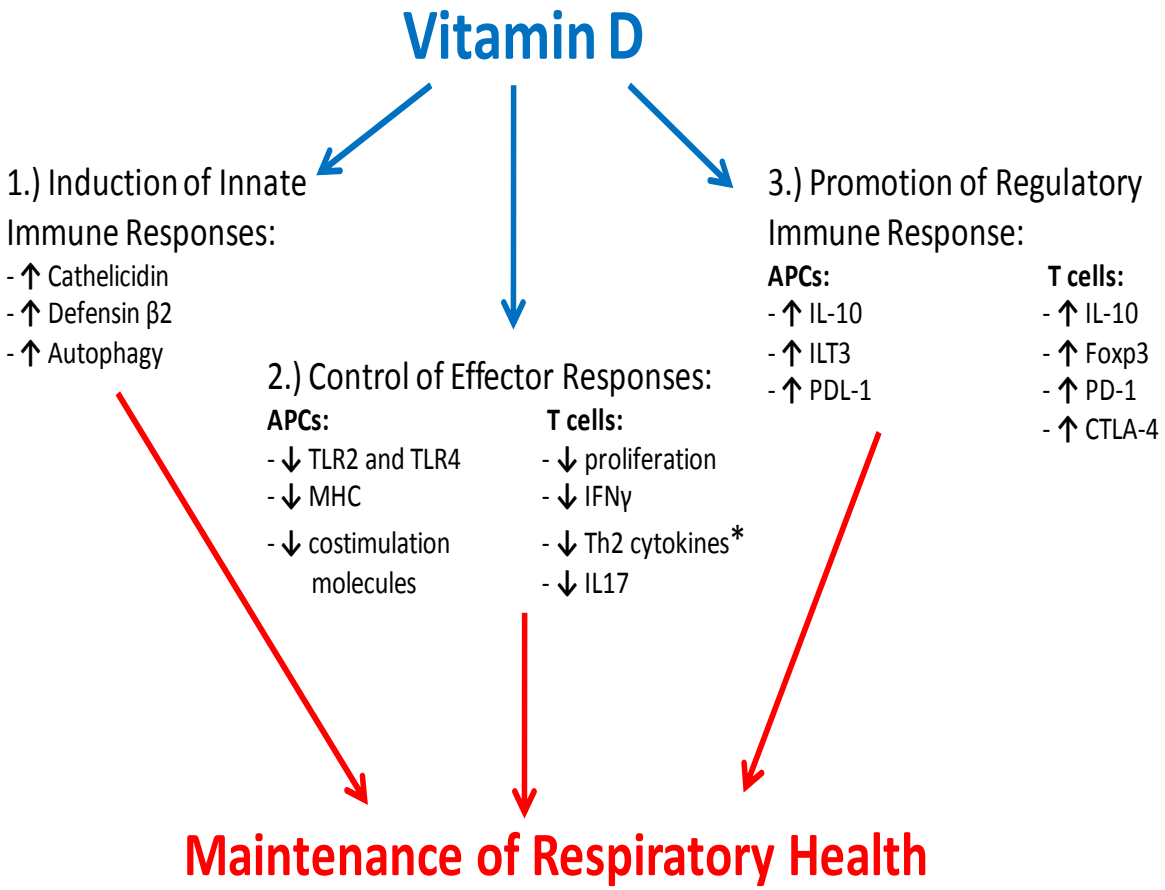


Figure 1-8 The role of vitamin D in the maintenance of pulmonary health.

Vitamin D has wide ranging immunomodulatory properties. Vitamin D effects on the innate immune system include induction of antimicrobial peptides, such as cathelicidn and defensin β2 and induction of autophagy. Vitamin D controls the effector CD4+ T cell response through decreasing expression of TLR molecules and costimulatory molecules on the antigen presenting cells. Additionally Vitamin D enhances regulatory T cell populations (IL-10+ and Foxp3+) as well as enhancing IL-10 production and inhibitory molecule expression on antigen presenting cells. * = this is still under investigation and is still considered controversial by some. Adapted from [312,313].

Vitamin D has been proposed to play a role in maintaining respiratory health in three ways as shown above: firstly by inducing innate immune responses which help with defence against respiratory pathogens; secondly by reducing inflammatory responses and finally by inducing regulatory cells [313,314]. Thus $1,25(OH)_2D3$ is proposed to

have broad anti-inflammatory effects on the innate and adaptive immune response within whilst still maintaining anti-microbial defence through synthesis of anti-microbial peptides.

1.4.3 Vitamin D effects on Innate Immune Responses

$1,25(OH)_2D3$ has been shown to play an important role in innate immunity in particular in inducing the antimicrobial peptides such as Cathelicidin, an essential innate component for combating *M.tuberculosis* [315-317]. This induction of Cathelicidin by $1,25(OH)_2D3$ also results in the induction of autophagy (process of the cells degrading its own cellular components via lysosomes) which is involved in killing the mycobacterium in *M.tuberculosis* infected cells [318,319]. $1,25(OH)_2D3$ has also been shown to upregulate the pattern recognition receptor Nucleotide-binding oligomerization domain containing 2 (NOD2) which when activated in the presence of bacteria leads to release of the antimicrobial peptide defensin β2 [320]. This is an anti-microbial peptide which has been shown to play an active role in combating bacterial infections like *Pseudomonas aeruginosa* [321]. *M.tuberculosis* has been shown to induce the expression of Matrix metalloproteinases (MMPs), which can degrade components of pulmonary extracellular matrix; however $1,25(OH)_2D3$ has been shown to significantly decrease the production of MMPs thus reducing destruction of the lungs [322].

1.4.4 Vitamin D effects on Adaptive Immune Responses

When airway epithelium and Dendritic cells are exposed to $1,25(OH)_2D3$, there is a reduction in the production of pro-inflammatory cytokines which can instruct the adaptive immune response. The predominant mechanism for this is the induction of IκBα, and subsequent inhibition of the transcription factor NF-κB, a key transcription factor for cytokine production [271,323]. IL-2 cytokine production is also believed to be

inhibited by 1,25$(OH)_2$D3 through inhibition of the transcription factor NF-AT [324,325]. 1,25$(OH)_2$D3 has also been shown to reduce the proliferation of $CD4^+$ T cells in culture [324]. It is well documented that 1,25$(OH)_2$D3 inhibits the production of the cytokine Inteferon γ (IFNγ) via inhibition of Th1 cell polarisation [324,326,327]; however it is less clear the effect that 1,25$(OH)_2$D3 has on Th2-cytokines. A study reported that 1,25$(OH)_2$D3 enhanced Th2 development and cytokine production from naive splenic CD4+ T cells cultured in the presence of irradiated APCs in an antigen-dependent manner [328]. Subsequent studies showed that when splenocytes were cultured in Th1- or Th2-skewing conditions (in the absence of irradiated APCs and peptides), 1,25$(OH)_2$D3 significantly inhibited the development of Th1 and Th2 cells from naïve CD4+ T cells in culture [327]. 1,25$(OH)_2$D3 inhibits the generation of Th2 cells in CD4+ T cells from cord blood (a good source of naïve cells), cultured in Th1 and Th2 skewing conditions [329]. However it has been suggested that the Th2 response to 1,25$(OH)_2$D3 may be non-linear as high and low 25(OH)D serum levels in a large human study (>7000), corresponded with elevated IgE concentrations [330]. Thus very high and low levels of 1,25$(OH)_2$D3 may enhance Th2-mediated disease.
More recently 1,25$(OH)_2$D3 has also been shown to inhibit Th17 cytokine production in murine autoimmune disease models, and recently in human $CD4^+$ T cells *in vitro* [331-333]. It was also shown that 1,25$(OH)_2$D3 can inhibit Th9 generation *in vitro* [333].

1.4.5 Vitamin D effects on Dendritic cells

1,25$(OH)_2$D3 has been reported to promote tolerance and regulation through inhibiting the maturation of Dendritic Cells (DCs) and promoting T regulatory cell (Treg) populations. 1,25$(OH)_2$D3 has been shown to prevent the maturation of DCs, resulting in reduced expression of the costimulatory molecules CD80, CD86, and HLA-DR and the maturation marker CD83 [334-336]. It has also been shown to induce the expression of the inhibitory receptor Immunoglobulin-like transcripts 3 (ILT3); these

effects of $1,25(OH)_2D3$ seem to selectively target myeloid DCs not plasmacytoid DCs [337]. $1,25(OH)_2D3$ also induces the expression of the chemokine CCL22 from myeloid DCs which is a chemoattractant for Tregs [337]. It has subsequently been shown that $1,25(OH)_2D3$ upregulates the expression of programmed death ligand 1 (PD-L1) on the DC cell surface which results in inhibition of T effector cells [338]. Overall, $1,25(OH)_2D3$ renders the DCs more immature and hence more tolerogenic, and also upregulates molecules on the DC cell surface that result in inhibition of effector T cells whilst promoting induction and recruitment of Tregs [334].

1.4.6 Vitamin D effects on Regulatory T cells

Early mouse studies demonstrated that $1,25(OH)_2D3$ alone or in combination with immunosuppressive drugs can enhance $CD25^{hi}$ Tregs in murine autoimmune models, and the induction of these Tregs ameliorated disease [339,340]. In humans, $1,25(OH)_2D3$ can induce a population of $Foxp3^+$ T cells, and this induction is dependent on the presence of IL-2 [341,342]. These $Foxp3^+$ Tregs also expressed high levels of Cytotoxic T Lymphocyte Associated Protein 4 (CTLA-4) [341]. A potential mechanism by which $1,25(OH)_2D3$ may enhance $Foxp3^+$ Tregs is through VDR binding to the Foxp3 promoter region and subsequent enhancement of Foxp3 expression [343].
As well as $Foxp3^+$ Tregs, we and others have shown that $1,25(OH)_2D3$ increases the frequency of $IL\text{-}10^+$ Tregs [344-347]. Subsequent studies demonstrated that human peripheral blood $CD4^+$ T cells polyclonally activated in the presence of $1,25(OH)_2D3$ promoted IL-10 secreting Treg cells. TLR9, was described as a biomarker of $1,25(OH)_2D3$ induced $IL\text{-}10^+$Treg and ligation of TLR9 with its agonist CpG oligonucleotide turned off IL-10 production, suggesting a control mechanism, whereby Treg function may be abrogated [346]. This may be of relevance, for example during infection, where the capacity to transiently block inhibitory function would facilitate a more effective immune response for clearance of the pathogen. Elimination of the

pathogen would lead to diminished TLR ligation, allowing restoration of the Treg response and thereby minimise tissue damage [348].

1.4.7 The steroid enhancing properties of 1,25(OH)$_2$D3

Glucocorticoids, such as dexamethasone have the capacity to enhance production of the cytokine IL-10 from CD4+ and CD8+ T cells in culture [185,187]. Dexamethasone inhibition of Th2-associated cytokines implicated in the pathogenesis of asthma is partially modulated through the upregulation IL-10. CD4+ T cells from SR asthma patients fail to produce IL-10 when cultured in the presence of dexamethasone, which is in contrast to CD4+ T cells obtained from SS asthmatics [349].

Further work demonstrated that 1,25(OH)$_2$D3 when used in conjunction with the steroid, dexamethasone induced a population of IL-10-producing regulatory cells which showed increased and more sustained IL-10 production in healthy donors. These IL-10+ cells were highly inhibitory in both an animal model of Experimental Autoimmune Encephalomyelitis (EAE) and in human *in vitro* suppression assays [347,350]. Importantly when 1,25(OH)$_2$D3 was added to cultures of cells from SR asthma patients it was able to overcome the defect in steroid-induced IL-10 synthesis. It was found that in healthy CD4+ T cells that although 1,25(OH)$_2$D3 alone did not increase the expression of GRα, it prevented the dexamethasone-dependent downregulation of GRα receptor [350]. Collectively this data provided a potential mechanism through which 1,25(OH)$_2$D3 enhanced dexamethasone-induced IL-10 responses. These results provided evidence for a proof of concept study where 4 healthy volunteers and 3 SR patients were administered Calcitriol (active from of Vitamin D; 1,25(OH)$_2$D3) orally at 2.5ng twice daily. Blood was taken at days 1, 3 and 7 after ingestion and cultured *in vitro.* Calcitriol treatment restored or enhanced responsiveness to dexamethasone in SR asthmatics for induction of IL-10 production *in vitro* [350]. This

data formed the basis of the clinical trial that forms part of this project, with the aim of the trial to investigate the capacity of calcitriol to restore the SR asthmatics clinical response to steroid.

1.5 Hypothesis and aims of the Thesis

The working hypothesis of the study was that SS and SR asthmatics had different phenotypes immunologically. The addition of $1,25(OH)_2D3$ *in vivo* has therapeutic potential through induction of regulatory cells and inhibition of inflammatory mediators. The aims of the project were therefore three fold:

- To investigate the lymphocyte and dendritic cell populations in SS versus SR asthma to identify any differences that could contribute to steroid responsiveness
- To identify the role of IL-17A in severe asthma and to investigate the potential use of $1,25(OH)_2D3$ in combating Th17-mediated disease.
- To identify the impact of cytokine milieu of $1,25(OH)_2D3$-induced Treg induction in culture; to identify potential mechanisms of Treg induction.

2. Materials and Methods

2.1 Patient/Donor Details

The clinical trial was approved by the Regional Ethics committee (REC) and the Medicine and Health Regulatory Authority (MHRA) with reference numbers; REC Ref Number: 08/H0804/84 and EudraCT Number: 2008-002244-42. Full written informed consent was obtained from all subjects. Patients were selected for the clinical trial if they met the criteria shown in Appendix I:

Experiments on healthy controls were from donors approved by Guy's Hospital Ethics Committee (09/H0804/77) and full written informed consent was obtained from all subjects. Atopy was defined by the presence of a positive wheal at 15 minutes after skin prick testing to ≥1 of a panel of aeroallergens performed with diluents (saline) and histamine controls.

2.2 Materials

RPMI and HBSS cell culture media along with other cell culture materials were obtained from Invitrogen Ltd. (Paisley, U.K.). 2-mercaptoethanol, Sodium Citrate and other general laboratory chemicals were obtained from Sigma Aldrich, (Gillingham U.K.) unless otherwise stated. Anti-mouse Ig, κ/negative control (FBS*) compensation particles set (Compbeads), FACs Flow and FACs lysing buffer were obtained from BD biosciences (Oxford U.K.).

2.3 Cell Culture

2.3.1 Media

All wash steps performed during isolation of peripheral blood cells were washed in HBSS supplemented with 2% FCS obtained from PAA Laboratories (Yeovil, U.K.) (referred to as 2% HBSS). Isolated cell populations were cultured in RPMI

supplemented with 10% FCS, 2mM L-glutamine and 50 µg/ml gentamycin (referred to as 10% RPMI).

2.3.2 Cell Purification and Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected by venepuncture into the anti-coagulant sodium citrate at a ratio of 10:1. Peripheral blood was diluted 1:1 in HBSS then layered at a ratio of 3:1 onto Lymphoprep® density gradient (Axis-Shield; Oslo, Norway) and centrifuged at 800g for 20 minutes at 18ºC. PBMCs were removed from the Lymphoprep®:plasma interface and cell pellet was obtained by centrifugation at 600g for 10 minutes at 4ºC. The cell pellet was washed twice in 2% HBSS (200g; 10 minutes at 4ºC). CD4+ T Cells were isolated by positive selection using CD4+ Dynal beads obtained from Invitrogen (Paisley, U.K.) CD4 beads were added at 50µl per 10^7 PBMCs for 20 minutes at 4ºC under continuous rotation. The sample was then placed on a magnet for 2 minutes and the supernatant removed. The bead-bound cells were washed by gently resuspending in 2% HBSS and then re-applying to a magnet and removing supernatant. This process was repeated six times then finally resuspended in 500µl of 2% HBSS. DETACHaBEAD® (Dynal) was added (CD4 beads 3µl: Detachabead 1µl ratio) and the cells were incubated for at least 30 mins at room temperature under continuous rotation. The sample was applied to a magnet and the supernatant containing the released, purified $CD4^+$ cells was transferred to a fresh tube. The beads were washed once in 2% HBSS to collect residual cells. Purity was routinely assessed with typical purity 97%, as seen below in Figure 2.1.

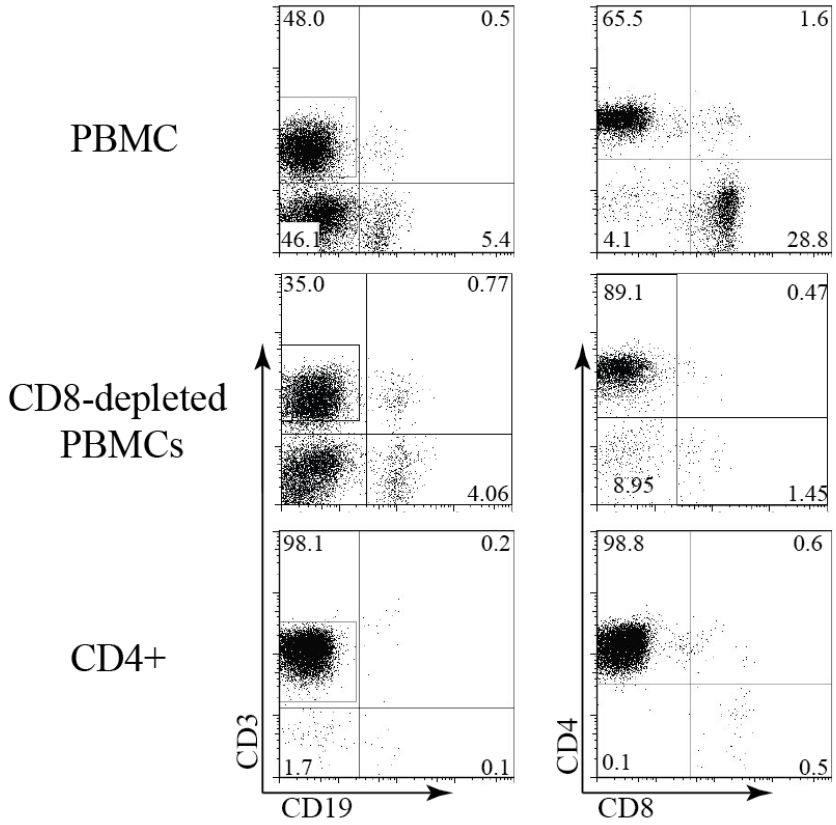


Figure 2-1 Purity of CD4+ and CD8-depleted cells isolated by bead-based selection

CD8-depleted PBMCs and CD4+ T cells were isolated from PBMCs by positive bead selection using antibody-coated Dynal® beads. Purity was assessed by surface staining using CD19-FITC, CD8-PE, CD3-PerCP, CD4-APC antibodies. 10,000 live cells were analysed for fluorescence by FACS Calibur. Values shown are indicative of the percentage of positive cells within the relevant quadrant.

For Treg and T effector isolation, CD4+ cells were isolated by negative selection using the Rosette CD4+ enrichment kit obtained from StemCell Technologies (Grenoble, France) from cones obtained from the National Blood Service. To Identify 'Treg' T cells ($CD25^{++}CD127^{lo}$) and 'effector' T cell ($CD25^{-}CD127^{hi}$) isolation was performed using a FACSARIA Flow Cytometer (BD Biosciences) and sort criteria was based on CD127 and CD25 surface staining.

2.3.3 Cell culture

Cells were counted using Trypan Blue to determine the number of viable cells. Cells were washed once in 2% HBSS (200g; 10mins 4^{o}C) then resuspended at 1x10^{6} cells/ml in 10% RPMI. CD4+ T cells were cultured for either one or two rounds of 7 day culture on an anti-CD3 coated plate (1µg/ml) with 50IU IL-2 obtained from Eurocetus (Harefield U.K.), with or without drugs as indicated were added to the culture. TGF-β, isotype control and anti-IL-10R antibodies were obtained from R&D systems (Abingdon, U.K.). 1α,25-dihydroxyvitaminD3 (1,25(OH)2D3) was obtained from BIOMOL Research Laboratories (Exeter, UK). POM-1 was obtained from Tocris Biosceince (Abingdon, U.K.).

2.4 Flow cytometric Analysis

2.4.1 Cell surface staining

Samples were incubated with fluorescently-labelled antibodies in the dark on ice for 45 mins. Whole peripheral blood samples were lysed using Facs lysing solution at working dilution (1:10), and incubated for 5 mins at room temperature and then centrifuged (200g; 5mins, 4^{o}C). All samples then were washed twice in FACs Flow (1ml; 200g; 5 mins at 4^{o}C). The samples were resuspended in 250-500µl of FACs Flow and subsequently run on the FASCalibur.

For clinical trial samples 80,000 total events were collected for lymphocyte phenotyping; 200,000 for DC phenotyping cells and 2000 (lineage negative, HLA-DR+) events were attempted to be gained for phenotyping makers on DCs. For other experiments 20,000 lymphocyte events were collected, except CellTrace proliferation experiments where 100,000 lymphocyte events were collected.

2.4.1.1 Antibodies

Antibody	Clone:	Obtained from:
CD4 (A780) CD8 (A780) CD25 (APC) CD39 (FITC) CD73 (APC) CD127 FoxP3 (FITC, PE) ILT3 (PE) mouse IgG1a, Isotype control (FITC) rat IgG2a, κ Isotype control (PE)	RPA-T4 RPA-T8 BC96 eBioA1 AD2 eBioRDR5 PCH101 ZM4.1 eBR2a	eBioscience (Hatfield U.K.)
CD3 (FITC, PerCp) CD4 (PE, APC) CD8 (PE, APC) CD11c (PE, APC) CD14 (FITC) CD16 (FITC) CD19 (FITC) CD20 (FITC)	SK7 RPA-T4 RPA-T8 B-ly6 M φP9 3G8 HIB19 2H7	BD biosciences (Oxford U.K.)

CD25 (FITC) CD45 (FITC) CD56 (FITC) CD80 (PE) CD86 (PE) HLA-DR (PerCp) IL-10 (PE, APC) IL-2 (PE, APC) phospho-STAT5 (A647)	M-A251 HI30 NCAM16.2 L307.4 2331(FUN-1) L243 JES3-9D7 MQ1-17H12 47/Stat5(pY694)	
BDCA-4 (PE, APC) BDCA-3 (APC)	AD5-17F6 AD5-14H12	Miltenyi Biotec (Bisley, U.K.)
GARP (APC) Helios (PE) LAP (PE)	LRRC32 22F6 TW4-2F8	Biolegend (Cambridge U.K.)

Table 2-1 A Table of antibodies used for flow cytometric analysis

2.4.2 Intracellular FoxP3 Staining

Samples were incubated with fluorescently-labelled antibodies as described above in 2.4.1. Samples were resuspended in 1ml of 1X Fixation/Permeabilisation solution and incubated for 45 minutes at room temperature in the dark. The samples were washed twice in 1X Permeabilisation buffer (1ml; 200g for 5 minutes at 4ºC). 5µl (0.25µg) of

FoxP3 antibody or 5µl of isotype control antibody was added to the appropriate tube and the samples were incubated for 45 minutes at room temperature in the dark. Following one wash in permeabilisation buffer and one wash in Facs Flow (1ml; 200g for 5mins at 4^{o}C); the cells were resuspended in FACS flow prior to FACS analysis.

2.4.3 Intracellular Cytokine Staining

Prior to staining, samples were restimulated for 4 hours with 5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin, with 2 µM monensin (Sigma-Aldrich) added for the final 2 hours. 2µM of 7-aminoactinomycin D (7AAD) was added to the samples (live dead stain), and incubated on ice for 15mins. Samples were then washed twice in ice cold FACs Flow (1ml; 200g for 5mins at 4^{o}C) and were resuspended in Cytofix/Cytoperm (BD Biosciences) for 20 mins at room temperature. Following one wash in FACs Flow (1ml; 200g for 5mins at 4^{o}C), samples were resuspended in 1ml of 1x Permeabilisation buffer (BD Biosciences) for 10 minute incubation at room temperature. Intracellular antibodies were then added and incubated with the samples at room temperature for 30 minutes. Finally, cells were washed once in 1x Permeabilisation buffer followed by once in Facs Flow (1ml; 200g for 5mins at 4^{o}C). Samples were resuspended in Facs Flow and either on a FACS Calibur or a FACS Canto flow cytometry machine (BD Biosciences).

2.4.4 Phospo-STAT5 Staining

An equal volume of CytoFix/CytoPerm Buffer (warmed to 37^{o}C) was added to the sample and this was incubated at 37^{o}C for 10 mins. Samples were washed twice in Facs Flow (1ml; 200g for 5mins at 4^{o}C), then 0.5ml of Perm Buffer III (chilled to -20^{o}C), obtained from BD Biosciences, was added to the samples whilst being vortexed. Samples were then incubated on ice for 30mins, then washed twice in FACs Flow (1ml;

200g for 5mins at 4^{o}C), and resuspended in 250µl of FACS Flow for analysis on a FACS Canto (BD Biosciences).

2.5 Proliferation Assays

2.5.1 CellTrace Violet

In some culture experiments, cell populations were labelled with CellTrace Violet obtained from Invitrogen. Cells were resuspended at 1x10^{6}/ml in HBSS with no protein and CellTrace Violet added at 10µM, cells were vortexed for 30 secs and then continuously mixed for 25 mins at RT. For the final 5 mins cold FCS was added, to stop the labelling reaction, make a final concentration of 10%. Cells were washed once in 2% HBSS (200g; 10mins at 4^{o}C), and then cultured as described in 2.3.4. Proliferation was assessed at Day 3, Day 7 and Day 14 by looking at the loss of fluorescence of CellTrace Violet using FACS Canto machine (BD Biosciences).

2.6 Cytometric Bead Array (CBA)

Cytokines were measured by Cytometric Bead Array (CBA) (BD Biosciences) according to a modified manufacturers protocol. The lower limit of detection was 1.5 pg/ml. Briefly, 50µl of sample was incubated with 20µl of cytokine specific-beads in a microtitre plate. The plate was shaken for (10mins; 80rpm), and then incubated for 3 hours (RT; in the dark). The plate is then centrifuged (200g; 5 mins) and washed twice in facs flow (200g; 5mins). 20 µl of Detection antibody is then added incubated for a further 2 hours (RT; in the dark). The plate is then centrifuged (200g; 5 mins) and washed with facs flow once (200g; 5mins) and resuspended in 150µl/well of facs flow, and cytokine profile is analysed using the BD Fortessa (BD Biosciences). Events were

collected on a BD Fortessa (BD Biosciences) and analysed using FlowJo (Treestar Inc.; Ashland, U.S.A.).

2.7 Isolation of Total RNA

RNA was isolated from the cell pellets using the QIAGEN RNeasy kit (QIAGEN Ltd., Crawley, UK) following the manufacturer's protocol. RNA was quantified using the Nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, U.S.A.) using ND-1000 software version 3.2.0. The purity and integrity of the RNA was assessed by A260/A280 spectrophotometric measurements.

2.8 Reverse Transcription to cDNA

250ng of RNA was reverse-transcribed into total volume of 30µl. 0.2 µg of random hexamers (Amersham Biosciences, Buckinghamshire, U.K.) and 250ng of RNA was heated to 70^{o}C for 10mins, and allowed to cool on ice. A master mix for each reaction, contained the following reagents: 1.5x reaction buffer, 40U of Ribolock RNase inhibitor, 200U of RevertAid H Minus M-MuLV and 1µM dNTPs obtained from Fermentas Life Science (York U.K.).

One unit of RiboLock RNase Inhibitor inhibits the activity of 5ng of RNaseA by 50%. One unit of the enzyme RevertAid H Minus M-MuLV Reverse Transcriptase incorporates 1nm of dTMP into a polynucleotide fraction (adsorbed on DE-81) in 10 min at 37^{o}C. Reactions were run on a Tetrad machine on the following programme:

25^{o}C	10 mins
42^{o}C	60 mins
70^{o}C	10 mins

4°C Hold

2.9 qRT-PCR

Transcriptional expression of target mRNA transcripts were determined by PCR amplification, quantified by 5'-nuclease assay using fluorescent labelled TaqMan® probes and analysed using an ABI PRISM 7900HT Sequence Detection System thermal cycler (Applied Biosystems, Foster City, USA). All primers/probes sets were purchased from Applied Biosystems. The sets used and the probe label are shown in Table 2.2.

Gene	Product Code	Probe Label
18S	Hs9999990_s1	VIC
5'-nucleotidase, ecto (CD73)	Hs01573922_m1	FAM
Cyp24a1	Hs00167999_m1	FAM
Ectonucleoside triphosphate diphosphohydrolase 1 (CD39)	Hs00969559_m1	FAM
FoxP3	Hs00203958_m1	FAM
IL-2	Hs00174114_m1	FAM
IL-10	Hs00174086_m1	FAM
TGFβ1	Hs99999918_m1	FAM
TGFβ2	Hs00234244_m1	FAM
TGFβ3	Hs01086000_m1	FAM

Table 2-2 A Table showing the probes used for qRT-PCR analysis

Reactions were performed in triplicate in 1 X TaqMan® Universal PCR MasterMix (Applied Biosystems) with typically 10ng reverse transcribed RNA, 0.5x gene specific primer/probe set and 0.25x 18S primer/probe set in a total volume of 10μl. Cycle parameters used:

Temperature (°C)	Time	
50	2 mins	
95	15mins	
95	15 secs	x45 cycles
60	30 secs	

2.9.1 qRT-PCR analysis

For each sample assayed, the Threshold Cycle (Ct) were determined for both the target gene and the 18S endogenous control, from the corresponding amplification plot according to guidelines from Applied Biosystems, using SDS software version 2.1 (Applied Biosystems). The Ct value for each reaction is the cycle number at the point where the amplification curve crosses the threshold of detection.

The data is expressed as arbitrary units. This is calculated by first normalising the data, subtracting the Ct value of the endogenous control from the target gene Ct (δCt), then the δCt of the reference or control sample was subtracted from the δCt of the other samples to give the δδCt. Finally, mRNA relative quantity of target genes was calculated using the equation: $2^{-(\delta\delta Ct)}$, which is expressed as RQ.

2.10 Flow Cytometric and Statistical Analysis

Flow data obtained from clinical trial samples and associated healthy donors were analysed using CellQuest Pro (BD Biosciences), samples acquired on a BD Canto or

BD Fortessa were analysed using FlowJo (Treestar Inc.). Data analysis was performed in Graphpad Prism version 5.00 for Windows obtained from Graphpad Software Inc. (San Diego, U.S.A.) using statistical tests detailed in the figure legend. To determine exact p values for ANOVA tests, statistics was also performed using SPSS Statistics Version 17.0 obtained from IBM SPSS Inc., (Portsmouth, U.K.).

3.*Ex vivo* phenotyping of Steroid Sensitive and Steroid Resistant Asthma

3.1 Introduction

3.1.1 Severe asthma

Severe asthma has been defined by the W.H.O. as uncontrolled asthma, with frequent severe exacerbations (or death) and/or failure to respond to medications and/or chronic morbidity [351] . Even with the treatment with high-dose of inhaled and/or oral steroids severe asthmatics have night and day symptoms, and low baseline lung function (<80% predicted FEV_1) which limit their daily activities [167,351].

3.1.2 Steroid Resistant (SR) Asthma

Within the severe asthma spectrum there is a group of asthmatics who are Steroid Resistant (SR), believed to be around 5-10% of asthmatics. These patients fail to show an improvement in lung function after treatment with a dose of oral steroids, unlike Steroid Sensitive (SS) asthmatics.

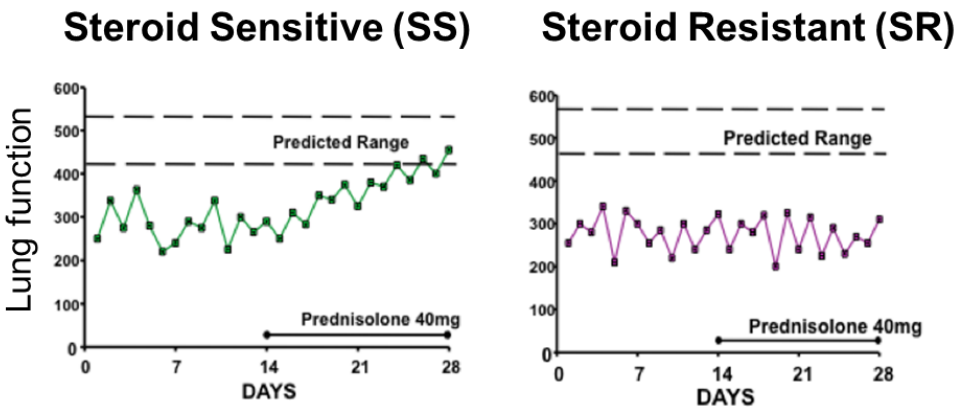


Figure 3-1 Representative lung function data of Steroid Sensitive and Steroid Refractory asthmatics after 2-weeks of Prednisolone

Representative lung function assessed in SS and SR asthmatics. Both SS and SR asthmatics were outside their predicted range as indicated by dotted lines. In the SS asthmatics a 2 weeks course of prednisolone restored their lung function to the predicted range, this was not observed in the SR asthmatics.

The first report of steroid resistant asthma was in 1968, where no reduction in peripheral eosinophilia after a course of oral glucocorticoids was reported [352]. Subsequently there has been a large body of research in the area of SR asthma and a number of mechanisms that contribute to SR asthma have been proposed including [183,353]:

- Reduced Histone Deacetylase (HDAC) expression and activity in SR asthmatics (HDAC together with glucocorticoid bound to the GR inhibits inflammatory gene transcription) [354-356].
- Mutations in the glucocorticoid receptor (GR) thus reducing the ability of the GRα to bind to target genes [357-359].
- Overexpression of GRβ; a dominant negative inhibitor of GR-α [360,361].
- Polymorphisms in the IL-10 gene resulting in reduced synthesis the anti-inflammatory cytokine IL-10 [222-226].
- NFκB and AP-1 is upregulated in environments high in oxidative stress (often seen in severe asthmatics and cigarette smokers). Components of AP-1 have been shown to suppress GRα function [353,362-364].
- Th2-associated cytokines as well as IL-2 (which are often seen overexpressed in Bronchioalveloar Lavage (BAL) of severe asthma patients) have been shown to be inhibitory of glucocorticoid action [365-368].
- A lack of steroidal induction of TIMP-1, resulting in a higher ratio of MMP:TIMP-1 thus enhanced tissue remodelling in the lung [369].

Phenotypically SR asthmatics have been shown to have a Th2-low disease. Instead severe asthma and steroid resistant disease has been proposed to be associated with an increase in Th17-associated cytokines (as will be discussed in the next chapter) [191,370,371]. Within the SR population there is an overrepresentation of smokers and people of black origin [183,189]. There is also epidemiological evidence associating

lower levels of serum 25(OH)D with a poor response to steroid treatment [302,305,372,373].

3.1.3 Steroid Enhancement by Vitamin D

Early studies from the host lab found that glucocorticoids increased production of the immunomodulatory cytokine IL-10 from CD4+ and CD8+ T cells in culture [185,187]. It is believed that inhibition of Th2 cytokines and particularly the induction of IL-10 contribute to the efficacy of steroids in Steroid Sensitive (SS) patients as the induction of IL-10 in response to glucocorticoids is impaired by comparison in Steroid refractory (SR) patients *in vitro* [349]. Further work demonstrated that the active form of vitamin D, $1,25(OH)_2D3$ (also known as calcitriol) when used in conjunction with the steroid, dexamethasone (Dex) induced a large population of IL-10-producing regulatory cells which showed increased and more sustained IL-10 production and were highly inhibitory in both an animal model of Experimental Autoimmune Encephalomyelitis (EAE) and in human *in vitro* suppression assays [347]. Following these results a proof of concept study was established of 4 healthy volunteers and 3 SR patients who were administered Calcitriol (active from of Vitamin D; $1,25(OH)_2D3$) orally at 2.5ng twice daily according to British National Formulary (BNF) guidelines. Blood was taken at days 1, 3 and 7 after ingestion and cultured *in vitro.* Calcitriol treatment restored or enhanced responsiveness to dexamethasone in SR asthmatics for induction of IL-10 production *in vitro* [350]. These results have led to the development of a clinical trial that began in April 2009. The aim of the clinical trial is to identify whether the capacity of calcitriol to restore steroid-induced IL-10 production in cultures of cells for SR asthma patients, correlates with clinical benefit, specifically improvement in lung function in SR asthma patients. The primary endpoint of the study is change of FEV_1 at baseline compared to the end of the treatment period. This clinical study hypothesizes that $1,25(OH)_2D3$ will enhance clinical responsiveness to systemic glucocorticoid

therapy in the SR patient group and was performed by Dr Alexandra Nanzer a clinical research training fellow in the lab.

We hypothesized that differences exist in lymphocyte and dendritic cell populations in the peripheral blood of SS versus SR asthmatics. The work presented in this chapter utilises the unique opportunity offered by this clinical trial to compare the frequency as well as total numbers of immune cell populations in the peripheral blood from SS and SR patients via performing directly *ex vivo* flow cytometric staining, focussing on lymphocyte populations including B cells, CD4+ and CD8+ T cells and Foxp3+ T cells, as well as dendritic cell phenotyping. The effects of 2-weeks of oral prednisolone treatment on these parameters were determined. Information on vitamin D status of the patients, assessed as serum 25(OH)D levels by Dr Peter Timms at the Homerton Hospital, London, allowed further analyses of the associations between Vitamin D status and immune cell frequency and phenotype.

3.2 Results:

A flow diagram of the design of the clinical trial can be seen below (Figure 3-2). Full information on design and patient entry criteria of the trial are presented in Appendix I.

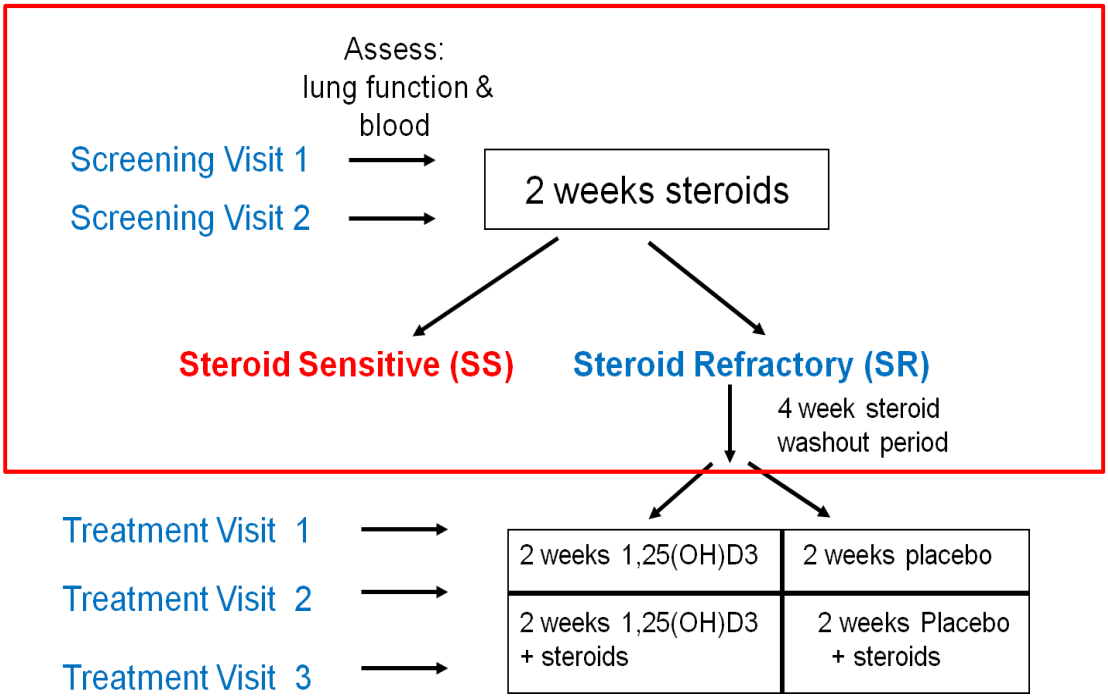


Figure 3-2 Schematic showing the Clinical Trial protocol

Severe asthmatics were recruited as described in Appendix 1. Briefly severe asthmatics were given prednisolone at Screening Visit 1 and returned two weeks later at Screening Visit 2, SS asthmatics were excluded from the trial and SR asthmatics were retained. After a four week wash out period the SR began the clinical trial where at Treatment Visit 1 the patient s were given either calcitriol ($1,25(OH)_2D3$) or placebo, at Treatment Visit 2 the patients were given Prednisolone and then they returned two weeks later for Treatment Visit 3. At each visit Lung function was performed and peripheral blood was isolated to perform immunological assessments. The red box around Screening Visit 1 and 2 highlights the focus of this chapter which is immunological assessments on SS and SR asthmatics pre- and post-steroids.

Due to the clinical trial being blinded at the time of writing, the focus of this chapter is comparison of SS and SR asthmatics pre- and post-steroids (as highlighted by the red box in Figure 3-2).

3.2.1 Patient characteristics.

The characteristics of the healthy control and severe asthmatics are shown below:

	Healthy controls	**Steroid Sensitive**	**Steroid Resistant**
Age (years)	41 (30-61)	48.5 (21-64)	53.3 (20-72)
Sex ratio (F/M)	3/7	8/4	11/14
FEV1 (L) * FEV1 (L) post steroids		1.65 (1.26-2.21) 2.08 (1.42-3.08)	1.88 (0.6-2.65) 1.83 (0.6-2.53)
FEV1 (% predicted) * FEV1 (%) post steroids		56% (45-76) 71% (54-87)	62% (22-79) 61% (22-80)
FeNo		31.6 (18-73)	26.5 (12-70)
Atopy (Y/N)	6/4	10/2	20/5
Race - Caucasian - African - Asian	 8 0 2	 9 3 0	 18 6 1
Average ICS dose (BDP equivalent) mcg		1133 (800-2000)	1200 (800-2000)

Table 3-1 Patient Characteristics – taken from Alexandra Nanzer's PhD thesis

Data shown for the severe asthmatics was from Screening Visit 1 (pre-steroids).

The table above gives an overview of the healthy controls and the severe asthma patients defined as either SS or SR. SR was defined as having less than 10% improvement in their lung function post a 2-week course of prednisolone as identified clinically by FEV_1. SS asthmatics (those who had >10% improvement in FEV_1) were excluded from the clinical trial (Treatment Visits 1 - 3). The asthma patients were recruited for screening for the clinical trial (Screening visits 1 and 2), of whom 12 were clinically defined to be SS and 25 were SR. There was no significant difference between age, race, sex ratio, atopic status and inhaled steroid dose between the SS versus the SR group. The only significant difference observed between the two cohort of patients was their lung function post 2-weeks of prednisolone, where SS had a significant improvement in their lung function (56% pre-steroids; 71% post-steroids) whereas SR did not (62% pre-steroids; 61% post-steroids) (post steroid FEV1 SR vs SS $p<0.001$ 95% CI 10.405-21.451, ANCOVA).

3.2.2 Healthy and Severe asthma donors had similar frequencies of lymphocytes.

As this was a unique opportunity to assess lymphocyte populations directly ex vivo in SS and SR asthmatics pre- and post-steroids, flow cytometry was performed. As part of the frequency of T and B cells (CD3+ and CD19+ respectively) were assessed in the peripheral blood, additionally CD4 and CD8 were used to differentiate the helper and cytotoxic T cell populations (within the CD3+ population). Additionally as DCs are known to be important for initiating or tolerising an immune response the frequency of pDCs and mDCs (as defined as being Lineage cocktail negative [CD3, CD14, CD16, CD19, CD20, CD56] and HLA-DR, and either BDCA-4 and CD11c respectively) was assessed whether there is different frequency of DC subsets in SS versus SR asthma. Additonally Foxp3+ Tregs were assessed through the following staining panel (CD3, CD25, CD127 and [intranuclear] Foxp3) as Foxp3 frequency and number has been

strongly associated with asthma severity and glucocortiocid treatment [172-174,176,178,188].

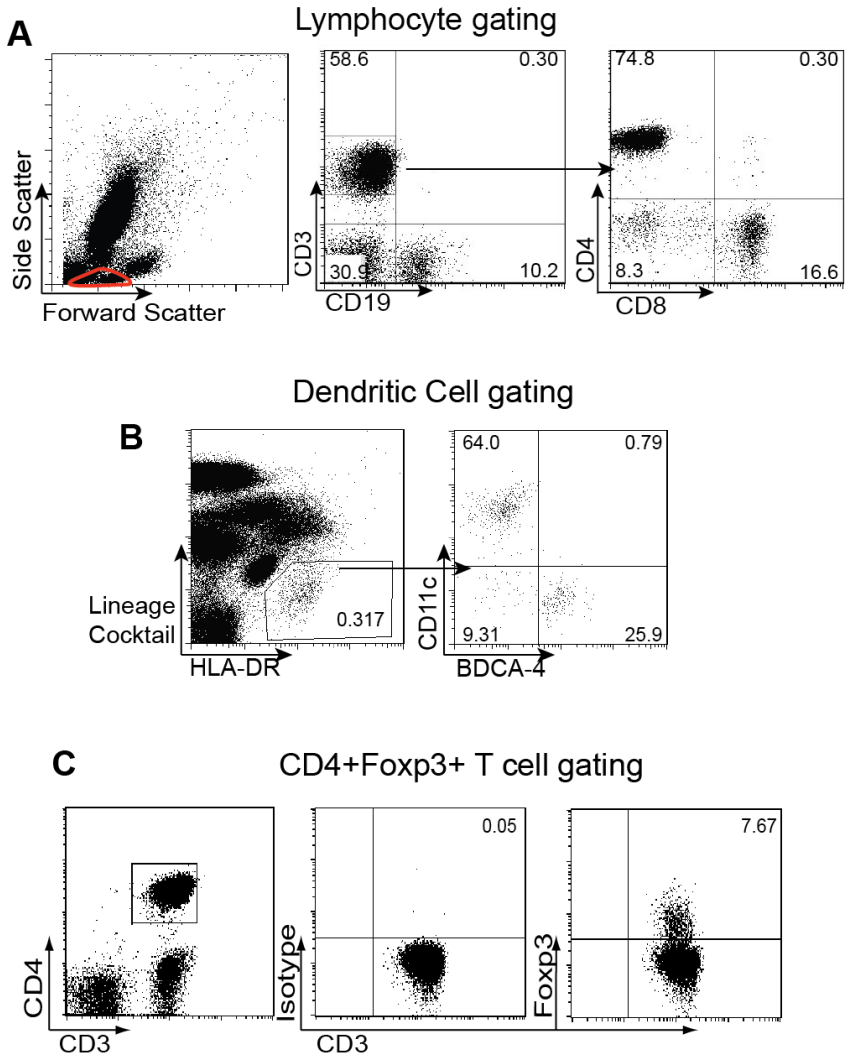


Figure 3-3 Example gating performed during ex vivo analysis of lymphocyte, dendritic cell and Foxp3+ populations

A, Representative dot plots showing lymphocyte gating based on forward and side scatter plots. CD3+ and CD19+ cells were identified within the lymphocyte population (highlighted in red), CD4+ and CD8+ cells were identified from within the CD3+ gate. **B,** Representative dot plots showing Dendritic cell gating, where DCs were identified as being lineage negative (Lin-)HLA-DR+. BDCA-4 and CD11c were used to identify plasmacytoid DCs and myeloid DCs respectively **C,** Representative dot plots showing CD4+Foxp3+ T cell gating. Foxp3+ expression

was assessed in the CD3+CD4+ lymphocyte gate, based on isotype control staining. Values shown represent the frequency of events in the quadrant.

The frequency of B cells (CD19+), T cells (CD3+), CD4+ T cells and CD8+ T cells and regulatory T cells (Foxp3+CD4+ T cells) were assessed by flow cytometry. Healthy non-asthmatic control donors were compared to the severe asthma patients, as seen below:

	Healthy	**Severe Asthma**	**p value**
CD19+ lymphocytes	7.32 (3.93-11.00)	8.68 (3.78-13.94)	0.30
CD3+ Lymphocytes	63.63 (56.34-74.25)	59.41 (40.20-73.88)	0.27
CD4+ T cells	62.15 (47.5-80.6)	64.96 (52.84-80.16)	0.48
CD8+ T cells	29.91 (16.11-44.91)	30.14 (17.48-42.49)	0.95
CD3+/CD19+ ratio	8.96 (4.48-18.81)	8.27 (4.12-14.22)	0.62
CD4+/CD8+ ratio	2.38 (1.08-5.07)	2.65 (1.25-4.59)	0.61
Foxp3+ Tregs	7.99 (6.76-10.11)	7.4 (4.96-10.8)	0.46
	n =10	n =37	

Table 3-2 Table showing the Frequency of Lymphocytes in the peripheral blood of healthy (non-asthmatic) donors and severe asthma donors.

CD19+, CD3+, CD4+ T cells, CD8+ T cells and Foxp3+ Tregs were gated as described in Figure3-3. Data shown as Mean ± 10-90% Percentiles, comparison between healthy and severe asthma assessed by unpaired t-test. Data shown for the severe asthmatics was from Screening Visit 1.

As can be seen above in Table 3-2, there was no significant difference in the frequency of B cells (CD19+), T cells (CD3+), CD4+ T cells and CD8+ T cells in the peripheral blood of healthy and severe asthma donors. Equally there was no significant difference between the ratio of B cells to T cells or the CD8+ to CD4+ T cells.

3.2.3 Severe asthmatics have an increased frequency of myeloid DC as compared to healthy donors.

DCs were identified as being Lineage negative (CD3, CD14, CD16, CD19, CD20 and CD56) HLA-DR+ cells. DCs were further characterised as myeloid and plasmacytoid on the basis of CD11c and BDCA-4 respectively. The frequency of the DC populations was assessed in healthy (non-asthmatic) donors and severe asthmatic donors:

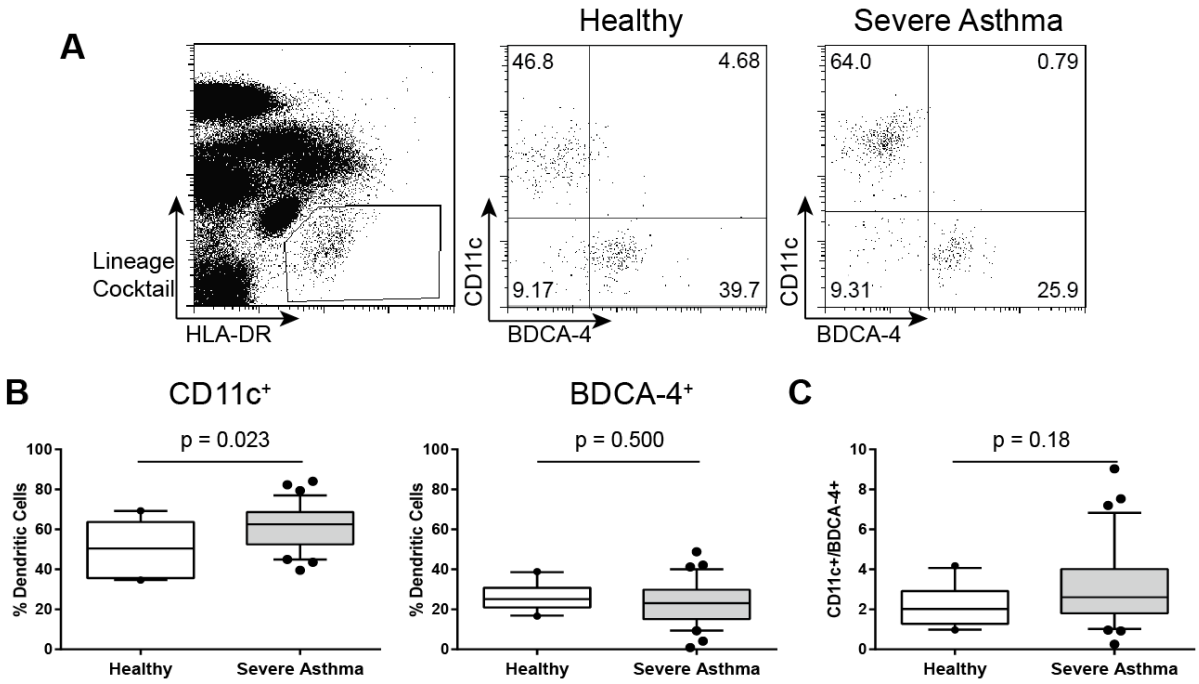


Figure 3-4 The frequency of Myeloid and Plasmacytoid DC in healthy and severe asthma donors

A, Frequency of CD11c+ and BDCA-4+ cells within the DC gate (Lin-HLA-DR+) **B,** Ratio of CD11c+ to BDCA-4+ DCs in healthy (n=10) and severe asthma donors (n=33). **B**, Healthy versus severe asthma assessed by unpaired t-test. Data shown from severe asthma patients was from Screening Visit 1.

As can been seen from Figure 3-4 above, there is significantly more CD11c+ (myeloid) DC in the peripheral blood of severe asthmatics as compared to healthy donors, whereas there is no significant difference between BDCA-4+ (plasmacytoid) DCs. In

Figure B there is a trend towards an increased ratio of CD11c+/BDCA-4+ cells in the severe asthma patients as compared to healthy controls.

3.2.4 Comparison between healthy and severe asthmatics after 2-weeks of steroids

To determine if a two-week course of steroids changes the frequency of lymphocytes and DCs from healthy donors, a comparison between healthy donors and severe asthmatics post-steroids was performed:

	Healthy	Severe Asthma	p value
CD19+ lymphocytes	7.32 (3.93-11.00)	11.77 (4.41-19.11)	0.01
CD3+ Lymphocytes	63.63 (56.34-74.25)	51.11 (26.0-76.2)	0.20
CD4+ T cells	62.15 (47.5-80.6)	66.60 (53.0-79.26)	0.21
CD8+ T cells	29.91 (16.11-44.91)	28.65 (16.54-39.51)	0.70
CD3+/CD19+ ratio	8.96 (4.48-18.81)	5.05 (2.55-9.03)	0.002
CD4+/CD8+ ratio	2.38 (1.08-5.07)	2.71 (1.39-4.85)	0.49
Foxp3+ CD4+ T cells	7.99 (6.76-10.11)	5.69 (2.67-8.84)	0.004
	n =10	n =37	

Table 3-3 Frequency of lymphocytes in healthy and severe asthmatics post-steroids.

Data shown as Mean ± 10-90% Percentiles. Lymphocyte frequencies in healthy versus severe asthmatics assessed by an unpaired t-test. Severe asthmatic data shown was from Screening Screening Visit 2 (post-steroids).

As can be seen from the table above there was no significant differences in the frequency of CD3+, CD4+ and CD8+ T cells or in the ratio of CD4+ to CD8+ T cells in healthy and severe asthmatics post-steroids. There were significantly more B cells in

severe asthmatics post-steroids as compared to healthy donors, which resulted in a significant difference in the ratio of CD3+ T cell to CD19+ cells. There were significantly less Foxp3+ Tregs in severe asthmatics post-steroids as compared to healthy controls. These significantly changes are due to steroid usage as there were no significant differences in these lymphocyte populations pre-steroids.

3.2.5 Steroid Sensitive patients had a similar frequency and number of lymphocytes as compared to Steroid Refractory asthmatics at Baseline. The impact of steroid treatment *in vivo*.

As there was no difference between healthy and severe asthmatics the next aim was to identify if there were any significant differences between SS and SR asthmatics. The frequency of B cells (CD19+), T cells (CD3+), CD4+ T cells and CD8+ T cells were assessed by flow cytometry. The gating strategy shown in Figure 3-3A was used and lymphocyte populations were compared between SS and SR asthma as seen below in Table 3-3:

	Pre-steroids			Post-steroids		
	SS	**SR**	**p value**	**SS**	**SR**	**p value**
CD19+ lymphocytes	9.10 (4.89-14.05)	8.46 (3.56-15.12)	0.64	11.55 (2.93-20.90)	11.93 (4.60-19.03)	0.84
CD3+ Lymphocytes	58.57 (42.88-72.91)	59.86 (38.53-75.78)	0.74	53.37 (16.70-77.99)	49.94 (26.28-74.28)	0.62
CD4+ T cells	66.60 (55.85-81.79)	64.00 (48.10-79.45)	0.51	68.26 (53.98-82.10)	65.62 (50.05-79.29)	0.46
CD8+ T cells	28.90 (16.68-38.94)	30.87 (15.6-47.94)	0.60	27.38 (16.33-38.76)	29.40 (15.81-40.50)	0.52
CD3+/CD19+ ratio	7.386 (4.10-12.79)	8.74 (4.12-14.9)	0.30	5.40 (2.5-11.33)	5.12 (2.09-9.67)	0.81
CD4+/CD8+ ratio	2.58 (1.46-4.97)	2.688 (1.00-5.144)	0.84	2.83 (1.40-5.17)	2.61 (1.24-4.96)	0.65
	n = 13	n = 24		n = 12	n = 22	

Table 3-4 Frequency of Lymphocyte populations in SS and SR asthmatics was not different pre- and post-steroids

Data shown as Mean ± 10-90% Percentiles. Lymphocyte frequencies in SS versus SR asthmatics assessed by an unpaired t-test pre- and post-steroids. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

There was no significant difference in the frequency of CD19+ or CD3+ cells pre-steroid treatment or after 2-weeks of prednisolone between SS versus SR asthma patients (Table 3-3). The same was also seen with the frequency of CD4+ and CD8+ T cells. The ratio of CD3+/CD19+ and CD4+/CD8+ cells was not significantly different between SS versus SR asthmatics at Screening Visit 1 or 2 (Table 3-3).

	Steroid Sensitive			Steroid Resistant		
	Pre-steroids	**Post-steroids**	**p value**	**Pre-steroids**	**Post-steroids**	**p value**
CD19+ lymphocytes	9.34 (4.84-14.08)	12.11 (2.69-21.15)	0.09	8.08 (3.50-13.73)	11.58 (4.56-18.42)	0.003
CD3+ Lymphocytes	57.32 (42.2-70.60)	51.47 (14.56-78.15)	0.41	58.96 (37.64-76.25)	50.62 (25.39-74.49)	0.04
CD4+ T cells	67.30 (55.81-82.09)	68.26 (53.98-82.10)	0.54	63.41 (47.21-79.87)	65.79 (49.37-79.60)	0.31
CD8+ T cells	28.17 (16.32-38.97)	27.38 (16.33-38.76)	0.55	31.4 (14.39-49.06)	29.31 (15.57-40.58)	0.30
CD4+/CD8+ Ratio	2.58 (1.46-4.97)	2.827 (1.40-5.17)	0.34	2.64 (0.96-5.54)	2.64 (1.22-5.00)	0.49

Table 3-5 Frequency of Lymphocyte populations in SS and SR asthmatics pre- and post-steroids

Data shown as Mean ± 10-90% Percentiles (SS n=12; SR n=21). Differences in lymphocyte populations in SS and SR pre- and post-steroids were assessed by paired t-test. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

There was no significant difference in the frequency of CD3+CD4+ or CD3+CD8+ T cells in SS or SR asthmatics post-steroids. There was no significant difference in SS asthmatics post-steroids, but there was a significant reduction in the frequency of SR CD3+ cells (SS p=0.41; SR p=0.04). There was a significant increase in the mean frequency of CD19+ cells post steroid treatment in both SS and SR asthmatics (SS p = 0.09; SR p = 0.003). Thus due to the increase in the frequency of CD19+ cells in both SS and SR asthmatics post-prednisolone the ratio of CD3+ cells to CD19+ cells was also significantly changed post-steroids (Figure 3.5).

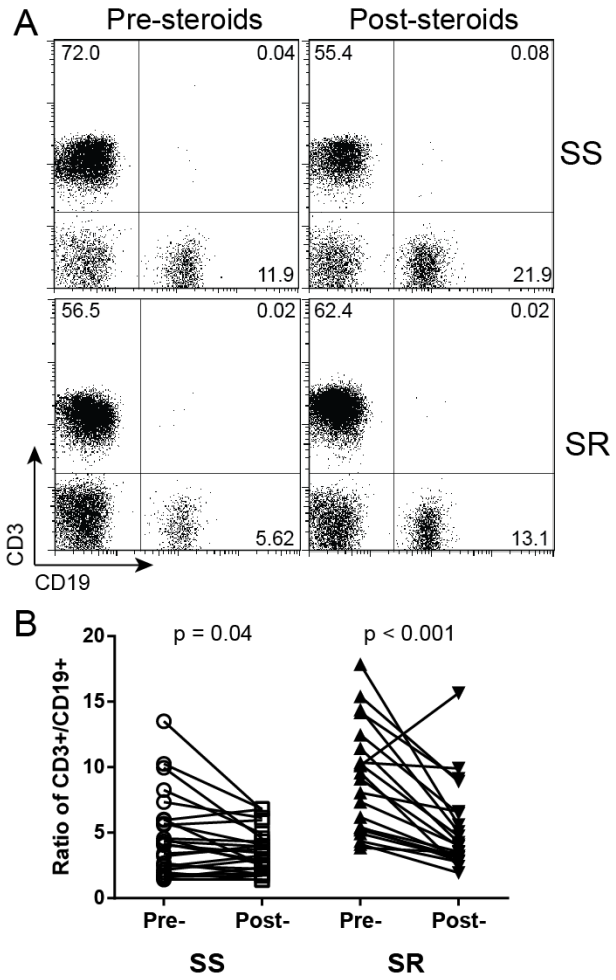


Figure 3-5 The ratio of CD3+ to CD19+ cells in the peripheral blood of severe asthmatics pre- and post- steroids

A, representative CD3 and CD19 staining in the lymphocyte population pre-steroids (right) and post-steroids (left) in SS (top) and SR (bottom) **B,** The ratio of CD3+ to CD19+ cells in the peripheral blood of SS (open shapes) and SR (closed shapes) pre- and post-steroids. **B,** CD3+CD19+ ratio in SS and SR asthma pre- and post-steroids assessed by paired t-test. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

There is a significant decrease in the ratio of CD3+ to CD19+ cells in the peripheral blood of severe asthmatics (Figure 3-5B), the lower ratio meaning that there were more CD19+ cells as compared to CD3+ cells post 2-week course of Prednisolone. This is most likely due to the increase in the frequency of CD19+ lymphocytes post-steroids as shown above in Table 3-4.

3.2.6 CD19+ lymphocyte numbers significantly increase post-prednisolone in both SS and SR asthmatics; CD3+ numbers decrease in SS asthmatic donors only.

Full blood counts were obtained at each time point in the clinical trial and this data was used in conjunction with the flow cytometric data to calculate the number of each population of lymphocytes in the peripheral blood of the severe asthma cohort, as seen below:

	SS	SR	p value
CD19+ lymphocytes	1.58 (0.71-2.85)	1.53 (0.51-3.07)	0.90
CD3+ Lymphocytes	10.41 (5.30-14.76)	11.38 (6.49-18.93)	0.57
CD4+ T cells	6.94 (3.63-11.13)	7.07 (2.51-14.58)	0.91
CD8+ T cells	2.98 (1.31-4.64)	3.23 (1.20-5.18)	0.71
	n =13	n = 22	

Table 3-6 Lymphocyte numbers in SS and SR asthmatics pre-steroids.

Data shown as Mean ± 10-90% Percentiles. Lymphocyte numbers in SS and SR asthma assessed by paired t-test pre-steroids. assessed by unpaired t-test. Data shown was from Screening Visit 1.

There was no significance difference in B cell (CD19+), T cell (CD3+) lymphocyte numbers between SS and SR asthmatics (Table 3-5). The same was also true with CD8+ and CD4+ T cells with no significant difference seen at baseline in SS versus SR.

The numbers of lymphocytes and also B cells, T cells and CD4+ and CD8+ T cells were compared in SS and SR asthmatics pre- and post-prednisolone.

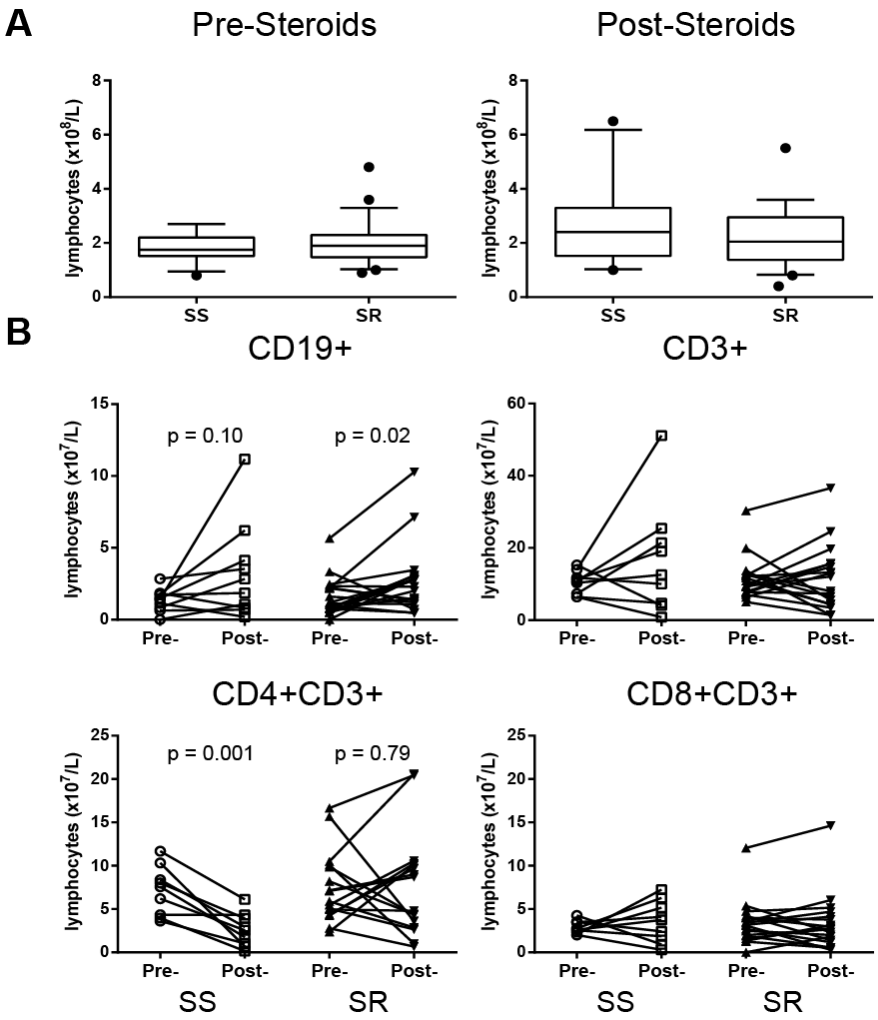


Figure 3-6 The number of lymphocytes in the peripheral blood of severe asthmatics pre- and post-steroids

A, The number of lymphocytes in the peripheral blood of severe asthmatics pre- and post-steroids **B,** The number of CD19+ (B cells), CD3+ (T cells), CD4+ T cells and CD8+ T cells in the peripheral blood of SS (open shapes, n=9) and SR (closed shapes, n=18) pre- and post-steroids. **A,** Differences in lymphocyte numbers in SS and SR asthmatics assessed by unpaired t-test pre- and post-steroids and **B,** differences in lymphocyte numbers pre- and post-steroids assessed by paired t-test in SS and SR patients. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

There was no significant difference in the total number of lymphocytes in the peripheral blood of SS and SR asthmatics as determined by full blood count performed as part of

clinical assessment pre- and post-steroids (Figure 3-6A). In contrast the number of CD19+ lymphocytes was significantly increased in SR post 2-weeks of prednisolone ($p=0.02$), and a similar trend was seen in the SS asthmatics ($p = 0.10$) (Figure 3-6B). There was no significant difference in the number of CD3+ T cells pre- and post-steroids in both SS and SR asthmatics. There was a significant reduction in the number of CD4+ T cells in the peripheral blood of SS asthmatics post 2-weeks of prednisolone ($p=0.001$), whereas there was no significant difference seen in SR asthmatics. There was no significant difference in the number of CD8+ T cells in SS or SR (Figure 3-6B).

3.2.7 SR asthmatics have a higher frequency of myeloid DC as compared to SS at baseline.

As well as lymphocyte populations DC frequency was assessed. As described before in Figure 3-3B, DCs were identified as being Lineage negative (CD3, CD14, CD16, CD19, CD20 and CD56) HLA-DR+ cells and were further characterised by the cell surface expression of CD11c (myeloid; mDC) and BDCA-4 (plasmacytoid; pDC) on the DCs:

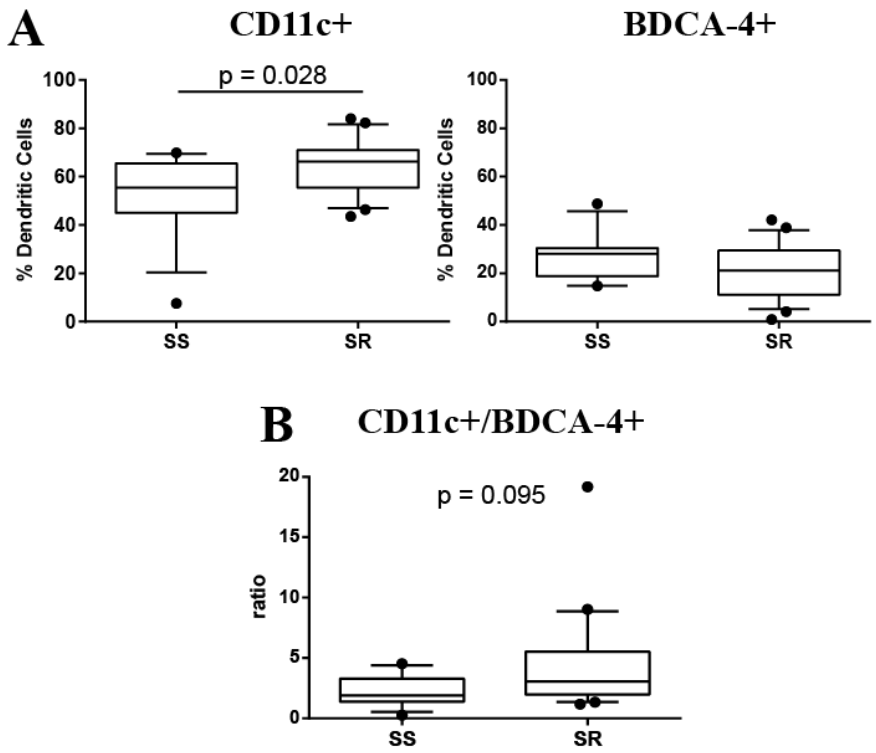


Figure 3-7 The Frequency of mDCs and pDCs in SS versus SR severe asthmatics.

DCs (lineage cocktail-HLA-DR+) were gated as shown in Fig 3-3. **A.** The frequency (%) of CD11c+ (mDCs) and BDCA-4+ (pDCs) **B,** the ratio of CD11c+/BDCA-4+ in peripheral blood of SS (n=13) and SR (n=21) asthmatics**. A,** differences in frequency of DCs in SS versus SR assessed by unpaired t-test. Values shown represent the frequency of events in the quadrant/gate. Data shown was from Screening Visit 1.

The SR asthmatics had a significantly higher frequency of CD11c+ DCs in the peripheral blood as compared to SS (p=0.028) (Figure 3-7A). There was no significant difference in the frequency of pDCs. There was a trend suggesting that there were more mDCs to pDCs in SR asthmatics as compared to SS as shown by the higher ratio of CD11c+/BDCA-4+ DCs in SR asthmatics (Figure 3-7B). Further phenotypic analysis of the CD11c and BDCA-4 DCs was performed by assessing the cell surface expression of CD86, ILT3 and HLA-DR. ILT3 is an inhibitory receptor expressed on DCs, and expression of ILT3 has been shown to be important for induction of Foxp3+

Tregs [334,374]. ILT3 was investigated on DC populations because it had been shown to be modulated by 1,25$(OH)_2$D3, and that ILT3 expression was important in ameliorating disease in patients who had psoriasis [334,337].

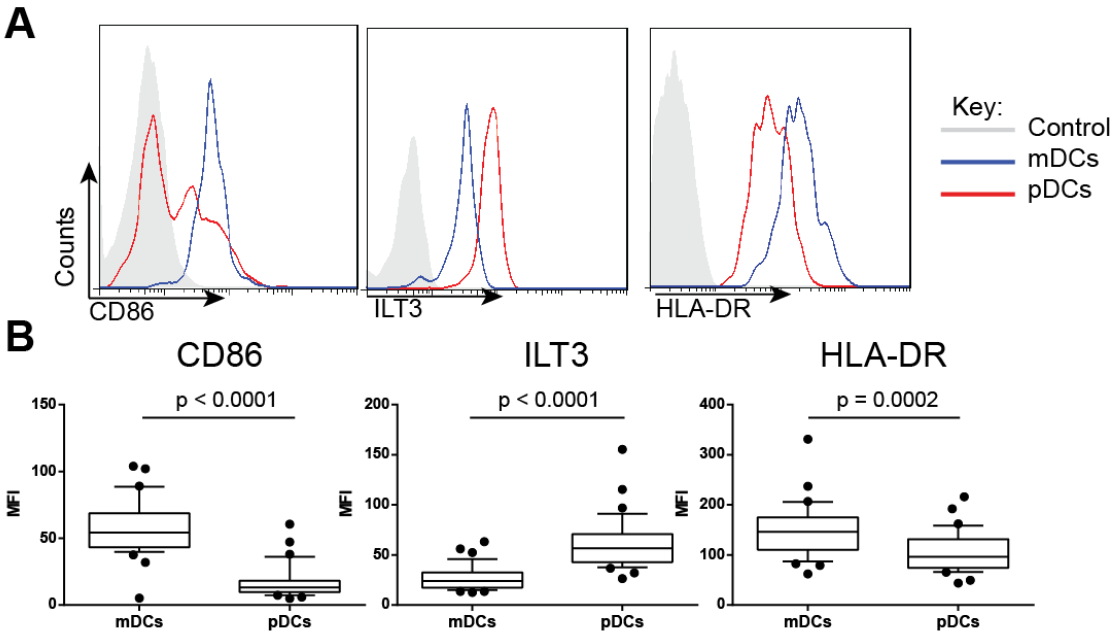


Figure 3-8 mDCs have higher expression of CD86 and HLA-DR but lower ILT3 as compared to pDCs.

DCs defined as being (lineage cocktail-HLA-DR+) were further characterised as CD11c+ (mDCs) and BDCA-4+ (pDCs) as shown above in Figure 3-4A **A,** Representative histograms of CD86 (left) ILT3 (middle) and HLA-DR (right) on mDCs (blue) and pDCs (red) as compared to FMO control staining (grey) **B,** cumulative data (severe asthmatics n=36). **B,** Expression of cell surface molecules on DCs in mDCs versus pDCs in all severe asthmatics assessed by unpaired t-test. Data shown was from Screening Visit 1.

Myeloid DCs, as defined by CD11c+ expression had significantly higher expression of CD86 and HLA-DR and lower expression of ILT3 as compared to pDCs. (Figure3-8).

3.2.8 The phenotype of mDCs and pDCs is the same at baseline (pre steroid) in SS and SR asthma patients

The expression of ILT-3, CD86 and HLA-DR on the surface of pDCs and mDCs was compared in SS and SR asthmatics.

	pDCs			mDCs		
	SS	SR	p value	SS	SR	p value
HLA-DR	105 (57.65-174.0)	112.2 (65.6-200.0)	0.66	165.5 (93.4-284.0)	161.6 (80.02-200.8)	0.91
ILT3	62.7 (33.06-106.2)	60.33 (37.17-83.03)	0.79	27.83 (13.54-47.78)	27.24 (15.52-50.77)	0.89
CD86	21.59 (6.25-57.86)	14.13 (7.829-30.26)	0.10	64.92 (34.2-88.69)	54.63 (41.51-92.01)	0.16
	n=14	n = 22		n=14	n = 22	

Table 3-7 Expression of HLA-DR, ILT3 and CD86 on pDCs and mDCs of peripheral blood obtained from SS and SR.

Data shown as Mean ± 10-90% Percentiles. Expression of HLA-DR, ILT3 and CD86 on pDCs or mDcs in SS versus SR assessed by unpaired t-test Data shown was from Screening Visit 1.

There was no significant difference between the expression of ILT3, CD86 and HLA-DR on pDCs and mDCs between SS and SR asthmatics at baseline (Table 3-6).

3.2.9 The frequency of total DCs and pDC significantly decreases post-prednisolone.

The effect of a 2-week course of prednisolone on DC frequency was investigated.

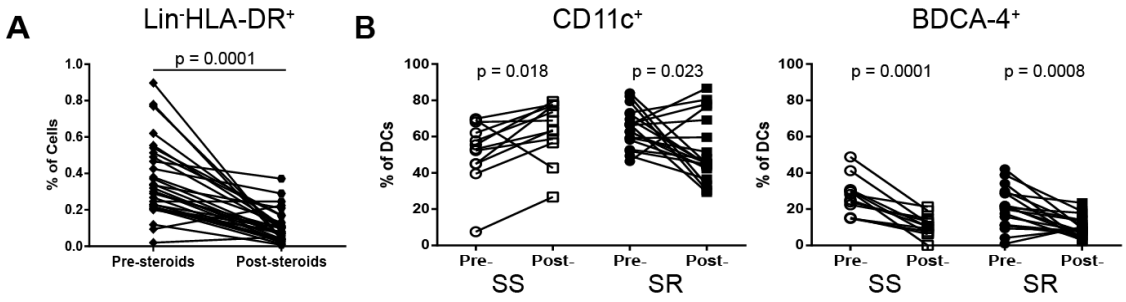


Figure 3-9 Dendritic cell frequencies significantly decrease post-steroids

A, The frequency of DCs (defined as being $Lin^{-}HLA\text{-}DR^{+}$) in the peripheral blood of all severe asthmatics pre- and post-steroids (n=31) **B,** The frequency of CD11c+ and BDCA-4+ DCs in the peripheral blood of SS (open shapes, n=12) and SR (closed shapes, n=19) pre- and post-steroids. **A,** frequency of DCs in all severe asthmatics pre- and post-steroids assessed by a paired t-test and **B,** differences in frequency of mDCs or pDC pre- and post-steroids assessed by paired t-test in SS and SR patients.

Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

A significant reduction in the frequency of all DC (defined as Lineage cocktail negative, $HLA\text{-}DR^{+}$) was observed from the peripheral blood of severe asthmatics post-steroids (pre-steroids mean = 0.412 (0.146-0.815); post-steroids mean = 0.094 (0.04-0.23) p=0.0001) (Figure 3-9A). The frequency of DC subsets was also investigated post-steroid treatment, and in SS asthmatics there was a significant increase in the frequency of mDCs (pre-steroids 52.06 (17.13-69.63); post-steroids 63.74 (31.49-78.87)), whilst conversely there was a significant reduction in the frequency of pDCs post steroids (pre-steroids 27.08 (14.80-46.50); post-steroids 10.97 (1.88-20.61)). In contrast in the SR asthmatics, there was a significant reduction in both mDCs (pre-steroids 64.75 (49.40-82.33); post-steroids 52.11 (30.40-80.49)) and pDCs (pre-steroids 21.00 (3.81-39.17); post-steroids 9.87 (2.53-19.28)) (Figure 3-9B).

3.2.10 The expression of HLA-DR and ILT3 on pDCs, and CD86 and HLA-DR on mDCs from SS asthmatics is significantly decreased post-steroids.

The expression of the markers CD86, Immunoglobulin-like transcript 3 (ILT3) and HLA-DR was assessed on pDCs from SS and SR asthmatics.

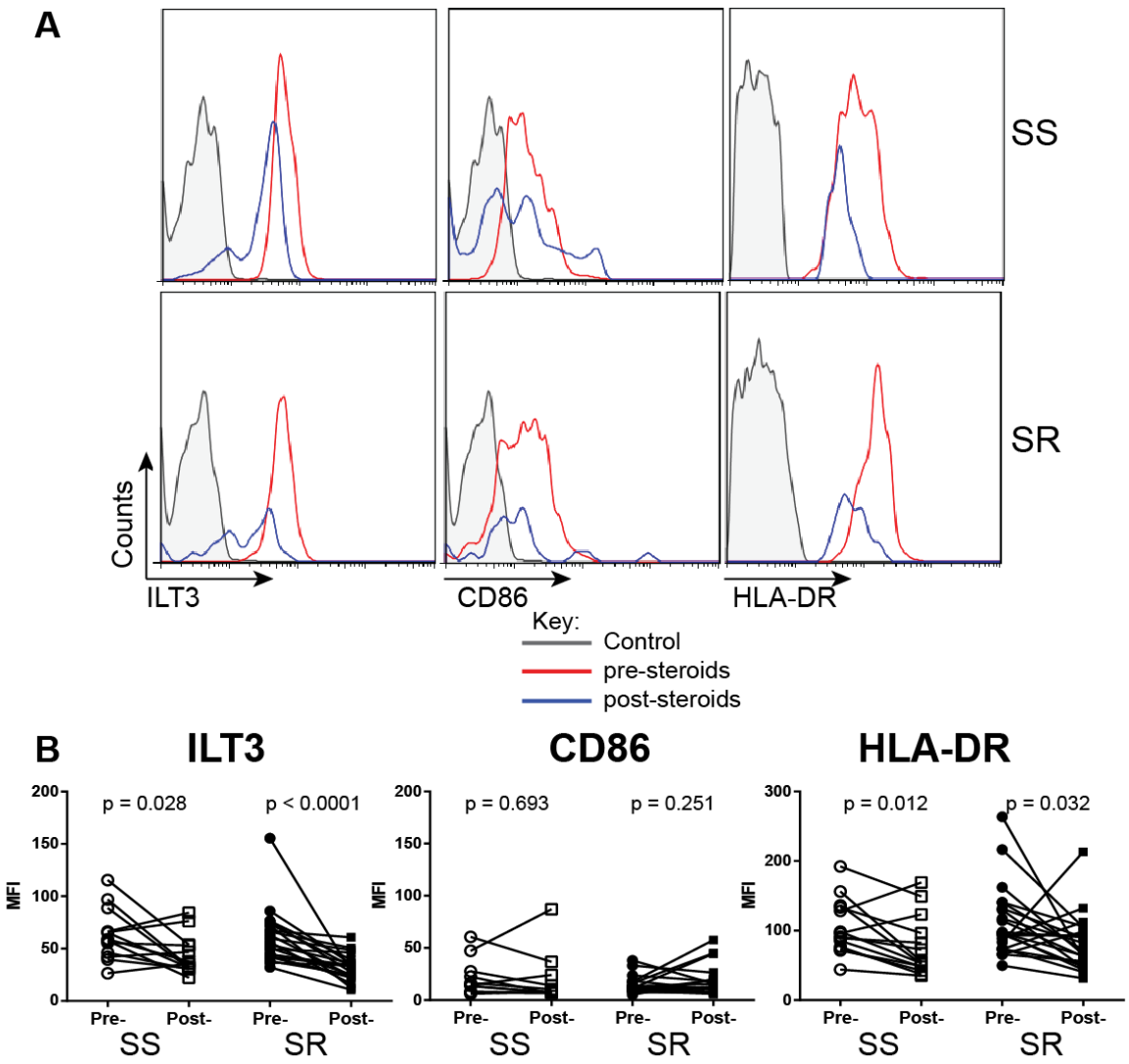


Figure 3-10 Expression of ILT3, CD86 and HLA-DR on pDCs in peripheral blood of SS and SR asthmatics pre- and post-steroids

A, representative histograms of ILT3 (left), CD86 (middle) and HLA-DR (right) expression on pDCs in SS (top) and SR (bottom) as compared to FMO control (grey) **B,** cumulative data of SS (open shapes, n=13) and SR (closed shapes, n=22) pre- and post-steroids. **B,** Expression of ILT3, CD86 and HLA-DR on pDCs pre and post-steroids assessed by paired t-test in SS and SR asthmatics. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

Although the data is limited by the low frequency of DCs observed post-steroids (Figure 3-9A) a significant reduction in the expression of ILT3 and HLA-DR on pDCs from both

SS and SR asthmatics post steroid treatment was observed. In contrast there was no significant difference in the expression of CD86 on pDCs post a 2-week course of prednisolone (Figure 3-10). ILT3, CD86 and HLA-DR expression was also assessed on mDCs pre- and post-steroids.

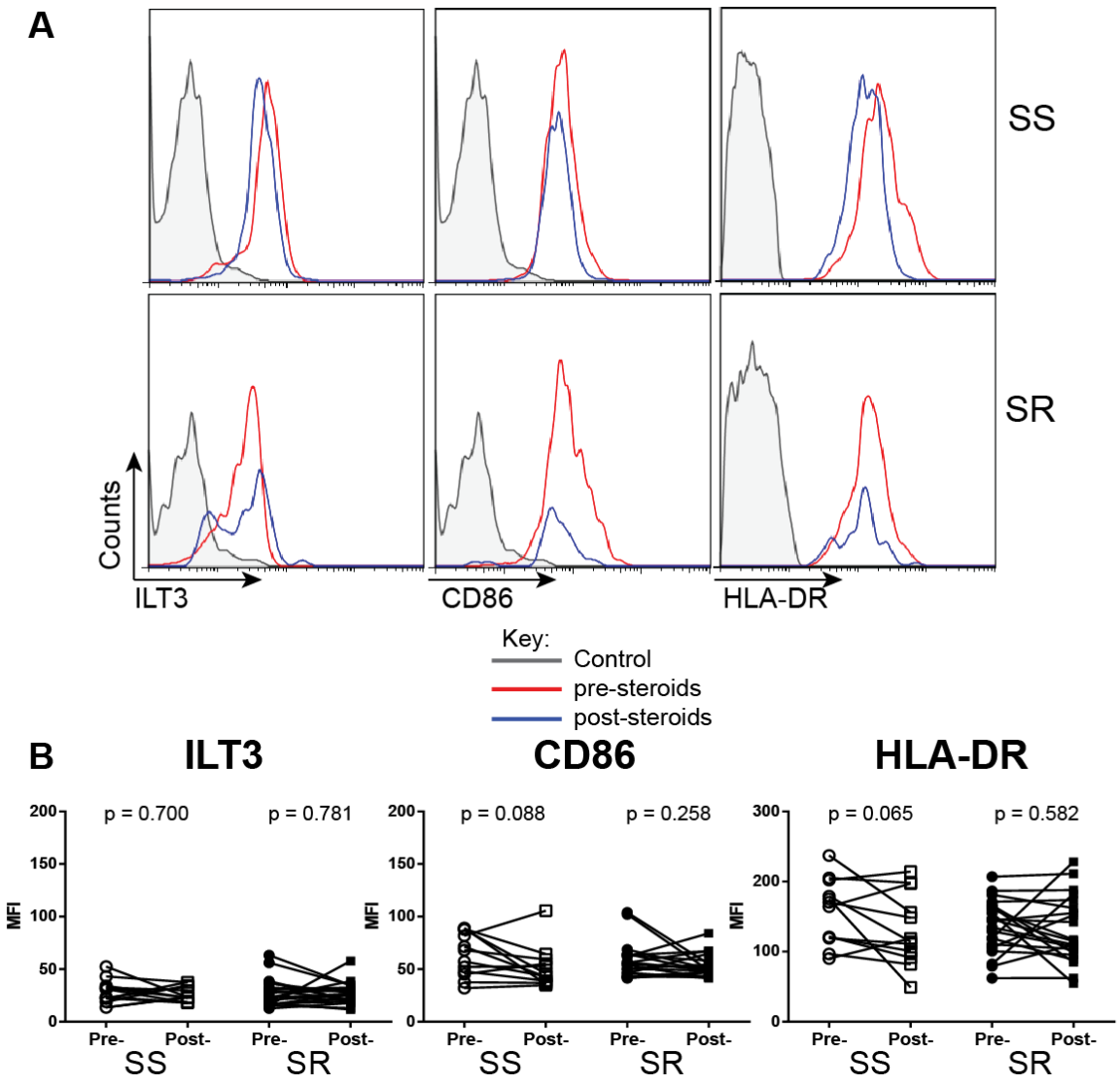


Figure 3-11 Expression of ILT3, CD86 and HLA-DR on mDCs in peripheral blood of SS and SR asthmatics pre- and post-steroids

A, representative histograms of ILT3 (left), CD86 (middle) and HLA-DR (right) expression on mDCs in SS (top) and SR (bottom) as compared to FMO control (grey) and **B,** cumulative data of SS (open shapes, n=13) and SR (closed shapes, n=22) pre- and post-steroids. **B,** Expression of ILT3, CD86 and HLA-DR on mDCs pre and post-steroids assessed by paired t-test in SS and SR asthmatics. Data shown is from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

There was no significant difference in the expression of ILT3 on mDCs post-steroids. There was a trend for reduction in the expression of CD86 and HLA-DR on the surface

of $CD11c^+$ DCs post-steroids in SS; this was not significant in SR asthmatics (Figure 3-11).

3.2.11 The frequency and number of Foxp3+ Tregs is lower in SR as compared to SS asthmatics pre- and post-prednisolone.

The frequency of Tregs, defined by expression of the transcription factor Foxp3, was investigated on CD3+CD4+ T cells as shown in Figure 3-3C. The cell surface markers CD127 and CD25 were also assessed to better indicate that the cells represented Foxp3+ Treg cells and to distinguish them from activated effector T cells.

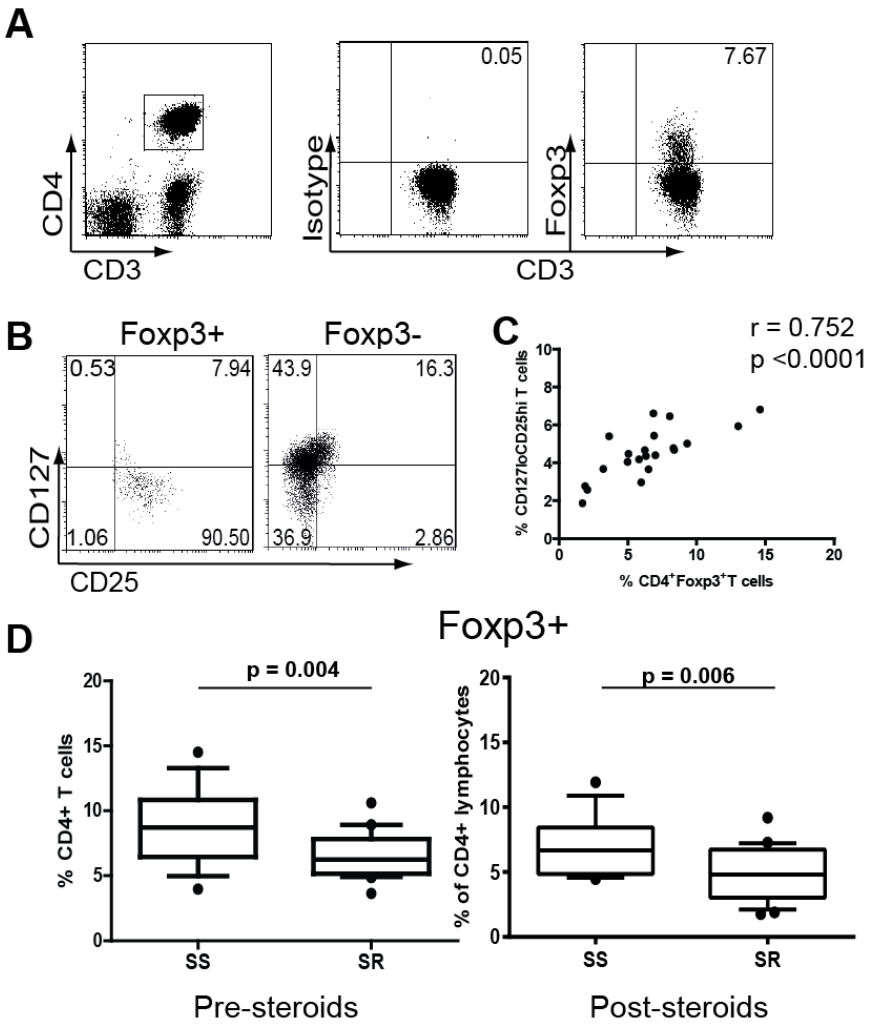


Figure 3-12 The frequency of Foxp3+ Tregs is lower in SR as compared to SS asthmatics pre- and post-steroids

A, Representative dots plots showing gating strategy for identifying Foxp3+ T cells (CD3+CD4+ lymphocytes) **B,** representative dot plots showing CD25 and CD127 staining within the Foxp3+ and Foxp3- population **C,** Correlation between frequency of Foxp3+ CD4+T cells and CD127loCD25hi CD4+ T cells **D,** Frequency of Foxp3+CD4+ T cells in SS (n=14) and SR (n=22) asthmatics Pre- and Post-steroids. **C,** correlation between % Foxp3+CD4+ T cells and % CD127loCD25hi CD4+ T cells assessed by Pearsons correlation test **D,** frequency of Foxp3+ CD4+ T cells in SS versus SR assessed by an unpaired t-test pre-steroids (left) and post-steroids (right). **A and B**, Values shown represent the frequency of events in the relevant quadrant. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

Foxp3+ Tregs were identified within the CD3+CD4+ lymphocyte population and Foxp3+ gating was based on isotype control staining (Figure 3-12A). To confirm that the gating strategy did contain 'real' Tregs, in sixteen of the severe asthma patients the cell surface markers CD127 and CD25 were used, as these markers had previously been identified to distinguish Foxp3+ Tregs from effector T cells [92]. Around 90% of the Foxp3+ CD4+ T cells were CD25hiCD127lo, whereas in the Foxp3- this was only 2.9% of the CD4+ T cell population (Figure 3-12B). The frequency of Foxp3+ Tregs positively correlated with the frequency of CD25hiCD127lo CD4+ T cells (Figure 3-12C). There was a significantly lower frequency of Foxp3+ Tregs in the peripheral blood of SR as compared to the SS asthmatics. This was also true after 2-weeks of prednisolone (Figure 3-12D).

The impact of Prednisolone on Foxp3+ Tregs frequency was assessed in SS and SR asthmatics.

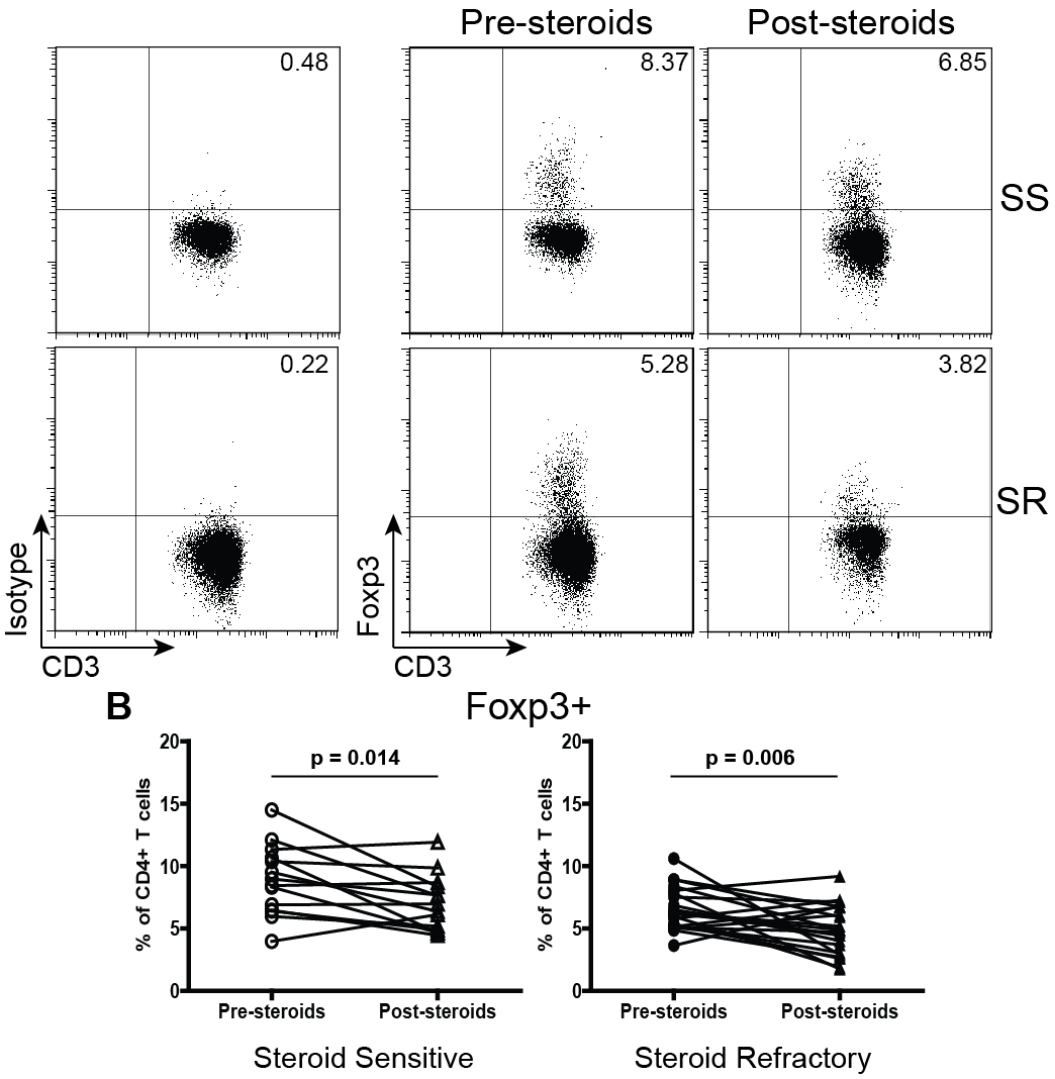


Figure 3-13 The frequency of Foxp3+ Tregs decreases post-steroids

A, representative dot plots from SS (top) and SR (bottom) donors, isotype control (left), pre- (middle) and post-steroids (right) **B,** Cumulative data showing the frequency of Foxp3+ Tregs pre- and post-steroids in SS (open shapes, n=14) and SR (closed shapes, n=22) asthmatics. **B,** differences in the frequency of Foxp3+ CD4+ T cells pre- and post-steroids was assessed by paired t-test in SS (left) and SR) right. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

In both SS and SR asthmatics there was a significant reduction in the frequency of Foxp3+ Tregs in the peripheral blood after 2-weeks of prednisolone (SS p=0.014; SR p=0.006) (Figure 3-13). The data from Figure 3-13 was used to calculate the numbers of Foxp3+ Tregs in the peripheral blood of the severe asthma patients, as seen below:

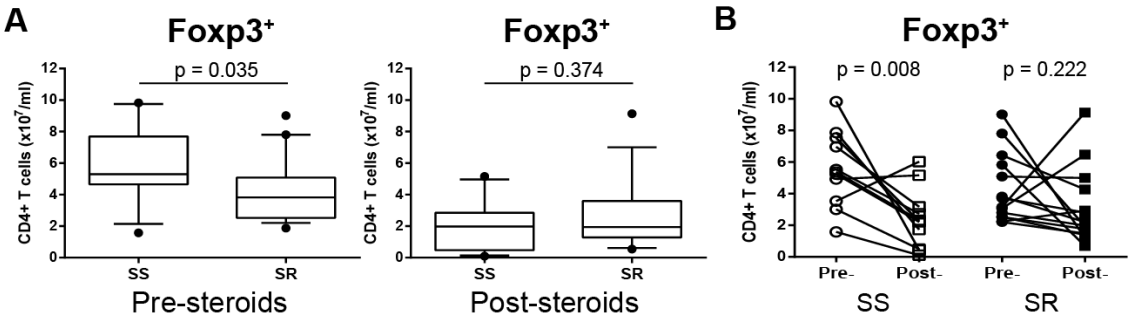


Figure 3-14 Foxp3+ Tregs pre- and post-steroids

A, The total number of Foxp3+ Tregs in the peripheral blood of SS (n=10) and SR (n=17) asthmatics pre-prednisolone (top left) and after prednisolone (middle) **B,** The total number of Foxp3+ CD4+T cells in the peripheral blood of SS (open shapes) and SR (closed shapes) pre- and post-steroids. **A,** number of Foxp3+ CD4+ T cells in SS versus SR assessed by unpaired t-test pre-steroids (left) and post-steroids (right) and **B,** number of Foxp3+CD4+ T cells pre- and post-steroids assessed by paired t-test in SS and SR. Data shown was from Screening Visit 1 (pre-steroids) and Screening Visit 2 (post-steroids).

At Screening Visit 1 before prednisolone, there were significantly lower numbers of Foxp3+ Tregs in the peripheral blood of SR as compared to SS (p=0.035), but post-prednisolone there was no statistical difference in the number of these cells between the 2 asthma cohorts (Figure 3-14A). The number of Foxp3+ Tregs in the peripheral blood of SS, but not SR, asthmatics was significantly reduced post-steroids (Figure 3-14B).

3.2.12 Severe asthmatics had significantly lower levels of serum 25(OH)D as compared to non-asthmatics.

The serum 25(OH)D levels (measurement of vitamin D status) was assessed at baseline (Screening visit 1) in the severe asthmatics and compared to the non-asthmatic donors.

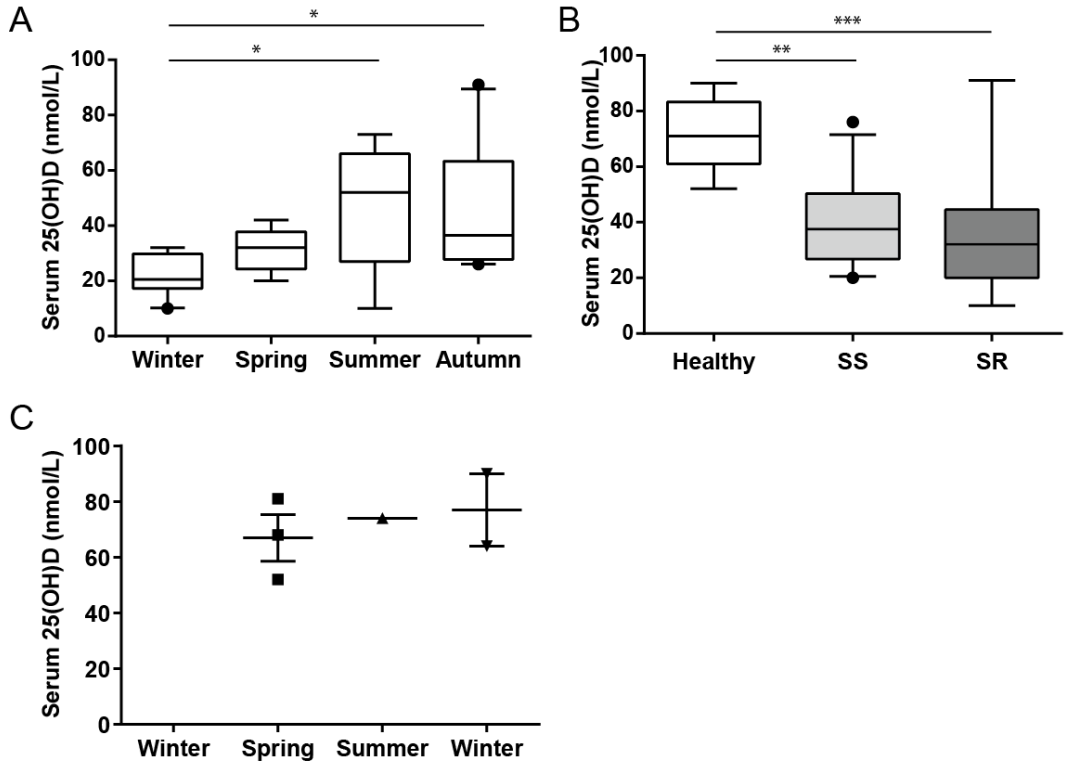


Figure 3-15 Serum levels of 25(OH)D changed with season and Severe asthmatics had significantly lower levels of serum 25(OH)D as compared to healthy controls.

A, Serum 25(OH)D levels in severe asthmatics throughout the year (Winter – Dec-Feb (n=10); Spring Mar-May (n=8); Summer – Jun-Aug (n=8); Autumn – Sept-Nov (n=10)) **B,** Serum 25(OH)D levels in Healthy (n=6) SS (n=12) and SR (n=23). **A,** differences in serum 25(OH)D through the seasons assessed by one-way ANOVA with Tukeys post-hoc test and **B,** differences in serum 25(OH)D in healthy versus SS versus SR assessed by one-way ANOVA with Tukeys post-hoc test. * p≤0.05 ** p≤0.01 *** p≤0.001. Data shown for severe asthmatics was from Screening Visit 1.

Even though the severe asthmatics were recruited all year round only two of the patients recruited had sufficient levels of vitamin D, defined as >75nmol/L 25(OH)D. Hence the majority of the severe asthma cohort were vitamin D insufficient (between 50-75nmol/L) or deficient (<50nmol/L) all year round. The patients recruited in Summer and Autumn had significantly higher levels of serum 25(OH)D as compared to Winter

and Spring (Figure 3-15A). Such seasonal variation was not seen in the few healthy donors that we have serum samples from (Figure 3-15C). There were significantly lower levels of serum 25(OH)D in the SS and the SR asthmatics as compared to healthy controls (Figure 3-15B).

3.2.13 Serum 25(OH)D positively correlated with the number of Foxp3+ Tregs.

To ascertain whether serum 25(OH)D had any influence on lymphocyte numbers, total lymphocyte numbers were assessed for correlation with serum 25(OH)D. No statistically significant correlations were observed (r=0.098; p=0.586). Also no correlation was observed between CD19+ cells (r=0.136; 0.4429), CD3+ cells (0.208; p=0.239), CD4+ T cells (r=-0.027; p=0.879) or CD8+ T cells (r=-0.063; p=0.722) with serum 25(OH)D. Foxp3+ Tregs numbers were also assessed for correlation with serum 25(OH)D.

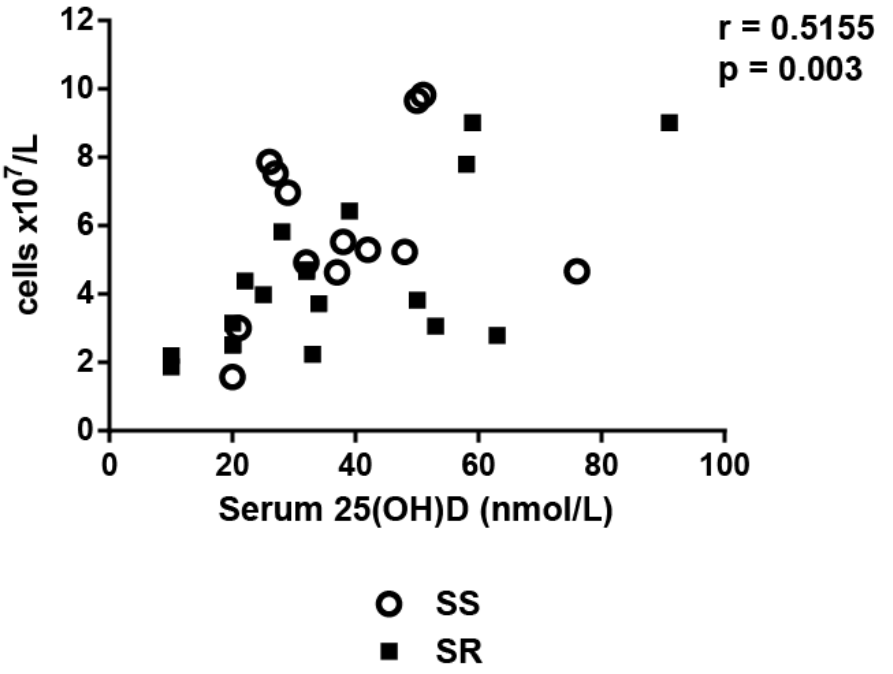


Figure 3-16 Positive correlation between serum 25(OH)D and Foxp3+ Tregs numbers in severe asthmatics.

Correlation between Serum 25(OH)D and number of Foxp3+ CD4+ T cells assessed by Pearsons correlation statistical test. SS asthmatics – open circles SR asthmatics – closed squares. Data shown was from Screening Visit 1.

Serum 25(OH)D positively correlated with the number of Foxp3+ Tregs in the peripheral blood of the severe asthma cohort. The individuals with the lowest number of Foxp3+ Tregs had the lowest serum 25(OH)D, and the converse was also true (Figure 3-16).

3.3 Discussion

The clinical trial provided a unique opportunity to directly assess *ex vivo* flow cytometry data comparing lymphocyte and dendritic cell populations in healthy subjects versus severe asthma patients, and also in SS versus SR. The main findings were (i) increased frequency of CD19 and associated CD19:CD3 ratios in severe asthmatics post-steroids; (ii) increased frequency of mDCs and (iii) decreased frequency of Foxp3+ Tregs in SR as compared to SS asthmatics and finally (iv) a significant positive correlation between the number of Foxp3+ Tregs and serum 25(OH)D. Summary of the SS versus SR asthma observations below:

	SS		**SR**	
	Frequency	**Number**	**Frequency**	**Number**
CD19+	=	=	=	=
CD3+	=	=	=	=
CD4+ T cells	=	=	=	=
CD8+ T cells	=	=	=	=
mDCs	-	nd	+	nd
pDCs	=	nd	=	nd
Foxp3+ Treg	+	+	-	-

Table 3-8 A summary Table showing the similarities and differences between SS and SR asthmatics

= means there is no significant difference; + means there is significantly more; – means there is significantly less between SS versus SR. nd = no data.

The effects of a 2-week course of prednisolone on the frequency and number of lymphocytes and dendritic cells from the peripheral blood of severe asthmatics are summarised below:

	SS		SR	
	Frequency	**Number**	**Frequency**	**Number**
CD19+	+	=	+	+
CD3+	=	=	-	=
CD4+ T cells	=	-	=	=
CD8+ T cells	=	=	=	=
mDCs	+	nd	-	nd
pDCs	-	nd	-	nd
Foxp3+ Treg	-	-	-	=

Table 3-9 A summary Table showing the impact of 2-weeks of Prednisolone on lymphocyte and dendritic cell numbers in severe asthmatics

= means there is no significant difference; + means there is significantly more; – means there is significantly less after 2-weeks of prednisolone. nd = no data.

There was no significant difference in the frequency of B cells and T cells in the peripheral blood of healthy subjects versus severe asthmatics at the start of the study and in severe asthmatics no significant difference in lymphocyte numbers pre and post steroids. Nevertheless after 2-weeks of steroids there was a significant increase in the frequency and total numbers of CD19+ cells in the peripheral blood of both SS and SR asthmatics. This resulted in a significantly lower frequency of CD3+ to CD19+ cells (CD3+/CD19+ ratio) in the severe asthma cohort after 2-weeks of steroids. With hindsight it would have been interesting to further investigate the phenotype of the

CD19+ T cells using cell surface markers such as CD5, CD24, CD27 and CD38 which are markers of B regulatory cells (Bregs) [61,375]. The increase in the frequency of CD19+ cell post-steroids has been observed previously [376].There were no significant changes in the numbers of CD3+ and CD8+ T cells post-steroids. Interestingly there was only a significant reduction in the number of CD4+ T cells in the peripheral blood of SS asthmatics and not SR. This data implies that a potential reason why the SR patients did not respond to steroids was due to the lack of effect on CD4+ T cell numbers. However due to the nature of the clinical trial it is not possible to determine if it is a defect in the patients response to steroids or due to a lifetimes use of steroids. In an independent study on healthy donors it was found that after a 2-week course of prednisolone there was no significant differences in the frequency of CD3+, CD4+ and CD8+ T cells [377], however a significant increase in all lymphocyte numbers (Total, CD3+, CD4 and CD8+ T cells) post-prednisolone was observed. This was believed to be due to leukocytosis, which is a well-known side-effect of glucocorticoids [377-379]. However, an increase in the number of lymphocyte populations was not seen in the severe asthma patients studied in this work. However, there was a significant increase in the neutrophil population post-steroids (data not shown; taken from Alexandra Nanzer's PhD thesis), suggesting that in this severe asthma cohort, leukocytosis only occurred within the neutrophil population.

A second major observation arising from this study was that there were significantly more mDCs in the peripheral blood of SR as compared to SS; this resulted in an increase in the ratio of mDC/pDC. Overall pDCs were found to have higher expression of ILT3 and lower expression of CD86 and HLA-DR as compared to mDCs. This data suggests that mDCs are more activated and are more ready to initiate immune responses, whereas pDCs due to the high expression of ILT3 and lower activation markers more tolerogenic. This supports a number of studies that have demonstrated that pDCs have regulatory capacity as they have been shown to be key in T-cell-

independent oral tolerance as well as prolonging graft survival in a mouse model of transplantation [380-382].

No difference was observed in the expression of ILT3, CD86 or HLA-DR on pDC and mDCs in SS versus SR. However due to the higher frequency of mDCs seen in SR asthmatics this suggests there are more DCs ready to initiate immune responses. The differential expression of ILT3 on pDCs versus mDCs is in contrast to work performed by Penna *et al*, where it was shown that both populations of DCs had similar levels of expression of ILT3 [334]. However in their study they isolated the DCs from a buffy coat first and then cell surface stained, and this isolation could have affected the expression of ILT3 and for this reason we stained directly ex vivo. In their study Penna *et al* used slightly different cell surface markers to differentiate mDCs and pDCs (BDCA-1 [CD1c] and BDCA-2 [CD303] respectively), and although these markers are supposedly redundant this may account for differences.

After 2-weeks of prednisolone there was a significant reduction in the frequency of all DCs, as well as pDCs, in the peripheral blood of severe asthmatics. This corresponds with other studies in autoimmune disease patients where prednisolone treatment caused a reduction in the frequency of plasmacytoid DCs in the peripheral blood. This is believed to be due to the fact that pDCs are susceptible to glucocorticoid-induced death [376,383,384], although we cannot also exclude effects on DC homing. Interestingly prednisolone did not have any significant effects on the activation and antigen presentation markers on DCs (CD86 and HLA-DR), and actually significantly decreased the expression of the tolerogenic marker ILT3 on pDCs. This is in contrast to earlier data which demonstrated in culture that steroids inhibit DC maturation and render them more tolerogenic. However those studies were performed on monocyte-derived DCs and thus was not looking at DCs directly isolated from the peripheral blood [385,386]. Furthermore, our data are the first to address *in vivo* effects in humans in severe asthma.

A third observation of interest from this study was evidence of no significant difference in the frequency of Foxp3+ Tregs in the peripheral blood of healthy donors as compared to severe asthmatics. This contradicts earlier findings which showed that mild asthmatics had a lower frequency of Foxp3+ Tregs as compared to healthy donors [172]. However, when the severe asthmatics were further characterised as being either SS or SR, there was a significantly increased frequency and number of Foxp3+ Tregs in the peripheral blood of SS as compared to SR asthmatics. Post-steroids there was still a significantly increased frequency of Foxp3+ Tregs in the peripheral blood of SS as compared to SR. However when total number of Foxp3+ Tregs was assessed there was no significant difference, which could be partially explained by the significantly lower number of CD4+T cells in the peripheral blood of SS as compared to SR.

After 2-weeks of prednisolone there was a significant reduction in the frequency of Foxp3+ T cells in both SS and SR. This is a contentious area in the literature at the moment as there are a number of studies that show that glucocorticoids in humans increase mRNA and protein levels of Foxp3 within CD4+ T cell populations *in vivo* [188,387,388]. There is also a large body of evidence in mouse models of allergic asthma and EAE, that administration of dexamethasone increases the frequency of Foxp3+ T cells [389,390]. Conversely, in other studies in healthy individuals and a paediatric asthma cohort, the opposite was observed and glucocorticoids reportedly decreased Foxp3+ Treg populations [377,391]. What is clear is that none of these earlier asthma papers studied severe asthmatic patients, but rather mild/moderate asthmatics (asthma was under control with only a requirement for low-dose inhaled steroids) and these patients will have a very different phenotype (mild asthmatics with no requirement for inhaled steroids) of asthma then the cohort of patients studied here (severe asthmatics on high inhaled steroids with the requirement of oral steroids during asthma exacerbations) [172,188]. The reduced frequency of Foxp3+ Tregs in the peripheral blood of the severe asthmatics post-steroids could be due to cells being

recruited to the site of inflammation i.e. to the lung environment, as it has previously been seen that inhaled steroids increased the number of $CD4^{+}CD25^{hi}$ T cells in the BAL of asthmatic children [174]. Although it would have been of interest to compare Foxp3+ Treg in both peripheral blood and the airways our study did not include this, not least because bronchoscoping severe asthma patients is difficult, and needs additional ethics approval from the clinical trial authority. Also it would have made recruitment to the trial much more difficult as it is an invasive procedure that would have taken additional time; hence we would have found it more difficult to recruit individuals.

All but two of the severe asthma patients recruited had insufficient if not deficient levels of serum 25(OH)D (mean: SS - 40.29nmols/L; SR – 33.76nmols/L). This was significantly different to the healthy donors who had a mean of 71.5nmol/L. However, the healthy donors represent a group of young, active people, and this 25(OH)D level does not reflect the serum levels of 25(OH)D seen in the normal U.K. population [279,392,393]. Hence this represents a weakness with the data as the healthy controls were not age and vitamin D status matched, and if this clinical trial was performed again I would have tried to match the healthy donors with the asthmatic patients. This data confirms earlier findings, including our own [373], that have shown that low levels of serum 25(OH)D correlate with disease severity and poor responsiveness to steroids [302,305,307,372,373,394], providing further evidence that vitamin D likely plays an important role in asthma control. A significant positive correlation seen between the number of Foxp3+ Tregs in the peripheral blood of the severe asthmatics and serum 25(OH)D ($r=515$; $p=0.003$) supports earlier findings in our lab correlating the frequency of BAL (airway) Foxp3+ cells and serum 25(OH)D, and other reports published correlating Foxp3+ Treg frequency and function with serum 25(OH)D in other human immune-mediated diseases [342,395,396]. These data collectively demonstrate an important role of vitamin D in modulatingTreg number and function and support earlier in vitro findings in our lab [342].

Interesting although the SR asthmatics characterised in this trial do not respond clinically to steroids, as assessed by changes in lung function (FEV_1), we are seeing immunological changes in the frequency of CD19+ and CD3+ cells as well as Foxp3+ Tregs. Thus suggesting that the immunological changes observed may not be important for the clinical response to steroids. However it also highlights that studying immune cell numbers and frequency in the airways would also be of considerable interest.

There are several limitations to the results generated from this trial; primarily the data observed was from the peripheral blood of the severe asthmatics. To gain a full insight into what is happening in these patients it would have been more informative to have studied BAL samples as well as the peripheral blood, because then we would have had more information about the tissue of interest. However access to BAL was not possible and was out of the remit of the trial and hopefully future trials will involve BAL samples as this would be very informative. Also it would have been very interesting to study the function of the Foxp3+ Tregs in these severe asthma patients, to determine if there was functional abnormality as well as there being a reduced frequency. However due to the limitations of peripheral blood obtained this was not possible.

This cross-sectional study demonstrates that the frequency of circulating Foxp3+ Tregs is significantly lower in steroid resistant than in SS asthmatic patients with comparable disease severity. The majority of patients with severe asthma had serum concentrations of 25(OH)D within the deficient range, and this strongly correlated with a paucity of Foxp3+ Tregs in the peripheral blood. These data suggest a strong functional correlation between vitamin D status, assessed as circulating 25(OH)D, circulating Foxp3+ Tregs numbers, and corticosteroid responsiveness in chronic asthma. They also provide important in vivo validation of in vitro studies with human cells and evidence from animal models suggesting that the vitamin D pathway plays an

important role in enhancing the frequency of the Foxp3+ Tregs compartment in humans.

4. Severe asthmatics have enhanced IL-17A production which is ameliorated by $1,25(OH)_2D3$. Potential role for CD39.

4.1 Introduction

Th17 cells have been demonstrated to be critical for defence against bacterial and fungal infections at mucosal surfaces [397]. Mice deficient in IL-17A or IL-17RA are highly susceptible to infection by mucosal pathogens such as *Klebsiella pneumoniae*, and *Candida albicans* [398,399]. It is believed that IL-17A is important for defence against pathogens through induction of inflammatory mediators such as IL-8 and G-CSF from airway epithelium cells, and IL-1β and TNFα from macrophages [400,401]. IL-17A-mediated activation of the innate immune system and neutrophil influx can be protective against infection but can also lead to damage of the surrounding tissues associated with immune pathology [402,403].

Originally Th17 cells and the associated cytokine IL-17A were implicated in steroid non-responsiveness in a murine model of allergic asthma [404]. It was shown *in vitro* and *in vivo* that transfer of Th17 or Th2 cells could both initiate airway hyperresponsiveness, and that only the Th2 cells could be inhibited with glucocorticoid [404]. Subsequently elevated IL-17A expression in human sputum or bronchial mucosal biopsies has been shown to correlate with bronchial hyperresponsiveness [405], granulocyte infiltration [406] and production of fibrogenic mediators by bronchial fibroblasts [407]. An elevated serum IL-17A concentration appears to be both a marker and an independent risk factor for severe asthma and steroid responsiveness [370,371,405-408].

In human studies it was shown that Th17-associated cytokines upregulate GRβ, a non-functional receptor for glucocorticoids, in human epithelial airway cells from severe asthmatics [409]. IL-22 another Th17-associated cytokine was also shown to have both protective functions with respect to airway infections, but also detrimental effects associated with immune pathology in the airways [410-412]. This was probably dependent at least in part on the location, cellular sources, cytokine milieu and timing of its expression [410-412]. It has been shown in an experimental model of allergic

asthma that IL-22 initiated allergic disease and that IL-22 enhances the inflammatory properties of IL-17A [56].

Several groups have pointed out striking links in population studies between vitamin D deficiency and increased asthma severity or poor responsiveness to anti-inflammatory medication [302,372,373,394]. In previous studies investigating the immunological basis of this relationship, we have shown that 1 α,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$) exerts immunomodulatory effects on T cells from patients with SR asthma, enhancing the impaired steroid-induced IL-10 response in these individuals [350]. Others have highlighted the capacity of $1,25(OH)_2D3$ to inhibit Th17 proliferation and function in mouse models of autoimmunity and also in human keratinocytes and T cells *in vitro* [331,413-415]. Conversely in a $VDR^{-/-}$ mouse model, where there is an absence of Vitamin D signalling, there is overproduction of IL-17A [416].

Controlling chronic inflammation including Th17-mediated disease whilst maintaining respiratory health, is the ideal aim for treating respiratory disease such as severe asthma. Regulatory T cells, and molecules that induce these populations such as vitamin D, represent an exciting prospect for limiting Th17-mediated disease whilst not being detrimental to respiratory health through induction of anti-microbial pathways [316,317,417]. Certainly it is known that Foxp3+ Tregs are important for controlling Th17-mediated diseases, in particular CD39+Foxp3+ T cells [138,418]. However the role of additional regulatory cell populations in controlling Th17-mediated disease has not yet been fully investigated.

In this chapter it was hypothesised that the severe asthma cohort secrete higher amount of Th17-associated cytokines as compared to healthy donors, and that $1,25(OH)_2D3$ may play a role in controlling the production of these cytokines. To achieve this Th17-associated cytokine production from cells isolated from the peripheral blood of severe asthma and healthy controls were assessed. Further to this

the role of dexamethasone and 1,25$(OH)_2$D3 in controlling Th17-associated cytokines were investigated in cells from healthy control subjects.

4.2 Results

4.2.1 Comparison of IL-17A and IL-22 production by CD8-depleted PBMCs from Steroid Sensitive (SS) and Refractory (SR) asthma patients compared to healthy controls *in vitro*.

Emerging epidemiological evidence suggests that SR asthmatics may make more IL-17A then less severe asthmatics and healthy controls [370,371]. Since asthmatic disease is believed to be CD4+ T cells-mediated disease and samples were limited in the amount of peripheral blood received from each severe asthma patient, CD8-depeleted PBMCs were utilised. CD8+ T cells were removed from the PBMC population as it has been historically used in the lab due to CD8+ T cells out competing CD4+ T cells when stimulated with anti-CD3 and IL-2 [185]. CD8-depleted PBMCs were isolated from the peripheral blood of healthy and severe asthma donors from the clinical trial and were investigated for Th17-cytokine production (patients characteristics can be found in Chapter 3 Table 3-1 and further information about the patient recruitment can be found in Appendix 1). Cells were cultured for 7 days with anti-CD3 and IL-2 in the presence or absence of drugs, and at day 7 PMA and Ionomycin was added for 4 hours, with the final 2-hours containing Monensin. Intracellular cytokine production was assessed by flow cytometry. Cells were also recounted at Day 7 and re-cultured at $1x10^6$/ml in the presence of anti-CD3 and IL-2 and in the absence of drugs for a further 48-hours to account for differential cell loss. Supernatants were collected and cytokine production was assessed by Cytometric Bead Array (CBA).

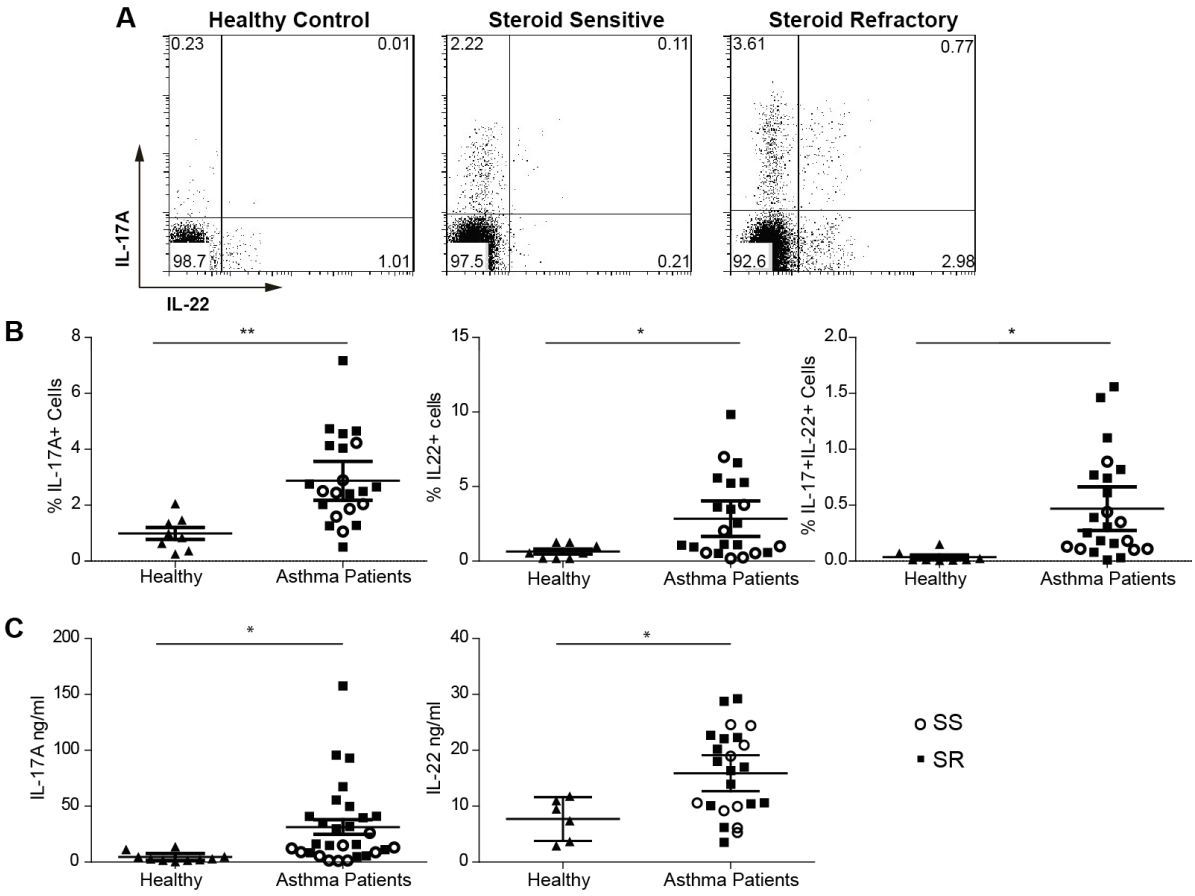


Figure 4-1 Severe asthma patients express higher levels of IL-17A and IL-22

CD8-depleted PBMCs were stimulated for 7-days with anti-CD3 and IL-2. Control and asthmatic cultures were assessed for IL-17A and IL-22 production by intracellular flow cytometry and CBA. **A,** Representative dot plots. **B,** Cumulative data of cytokine positive cells (healthy n=8; SS n=8 and SR n=14) and **C,** secreted cytokines (Healthy n=10; SS n=10 and SR n=14). **B, and C,** differences in the % of positive cells between healthy and severe asthmatics assessed by unpaired t-test, Severe asthma patients were from Screening Visit 1. * $p<0.05$; ** $p<0.001$. Open circles SS; closed squares SR; and closed triangles healthy controls.

This data is a combination of Dr Alexandra Nanzer's and my work. All healthy data is predominantly my work and the severe asthma data is predominantly Dr Nanzer's, however due to the nature and duration of the clinical trial there is a significant element of crossover.

Significantly greater percentages of IL-17A+ and IL-22+ expressing cells were observed in cultures from asthma patients, as compared to healthy control subjects

(Figure 4-1A & B). When patients were subdivided based on clinical responsiveness to glucocorticoids only SR patients showed a significant difference in IL-17A immunoreactivity (SR p=0.003; SS p=0.142) and IL-22 immunoreactivity (SR p=0.037; SS p=0.701), compared to control subjects (Figure 4-1B). Cells co-expressing IL-17A and IL-22 [56], are proposed to represent the most pathogenic population, and were significantly increased in all asthmatics and SR asthmatics compared to healthy control subjects (all asthmatics p=0.029; SR p=0.009). At seven days cells were also harvested and re-cultured at equal cell densities for 48 hours with anti-CD3 and IL-2 alone. Culture supernatants from asthma patients contained significantly higher quantities of secreted IL-17A and IL-22 as compared to healthy controls (Figure 4-1C). Production of IL-17A and IL-22 was significantly increased in all severe asthmatics as compared to controls (IL-17A: p=0.009; IL-22: p=0.036). Supernatants from SR had significantly more IL-17A as compared to SS (p=0.016), whereas culture supernatants from SS and SR patients contained comparable levels of IL-22.

4.2.2 Dexamethsone fails to inhibit whereas 1,25(OH)$_2$D3 decreases IL-17A production.

The severe asthma patients who secreted large amount of IL-17A in culture were on high doses of inhaled steroids (Table 3-3) and also had very low levels of serum 25(OH)D. In comparison the healthy controls had significantly higher levels of serum 25(OH)D and were not using inhaled steroids (Figure 3-14). This data implied that either/or the dose of steroids or the vitamin D insufficiency of the severe asthmatics may have influenced the high level of Th17-associated cytokine production. Hence the impact of steroids (dexamethasone) and 1,25(OH)$_2$D3 was assessed in CD8-depleted PBMCs from healthy controls *in vitro* to assess what impact these drugs had on Th17-associated cytokines. Cells were isolated from the peripheral blood of healthy donors and were investigated for Th17-associated and Th1- and Th2-associated cytokine

production. Cells were cultured for 7 days with anti-CD3 and IL-2 in the presence or absence of drugs, and at day 7 intracellular cytokine production was assessed by flow cytometry. Cells were also recounted at Day 7 and re-cultured at $1x10^6$/ml in the presence of anti-CD3 and IL-2 in the absence of drugs for further 48-hours, then supernatants were harvested and cytokine production was assessed by Cytometric Bead Array (CBA).

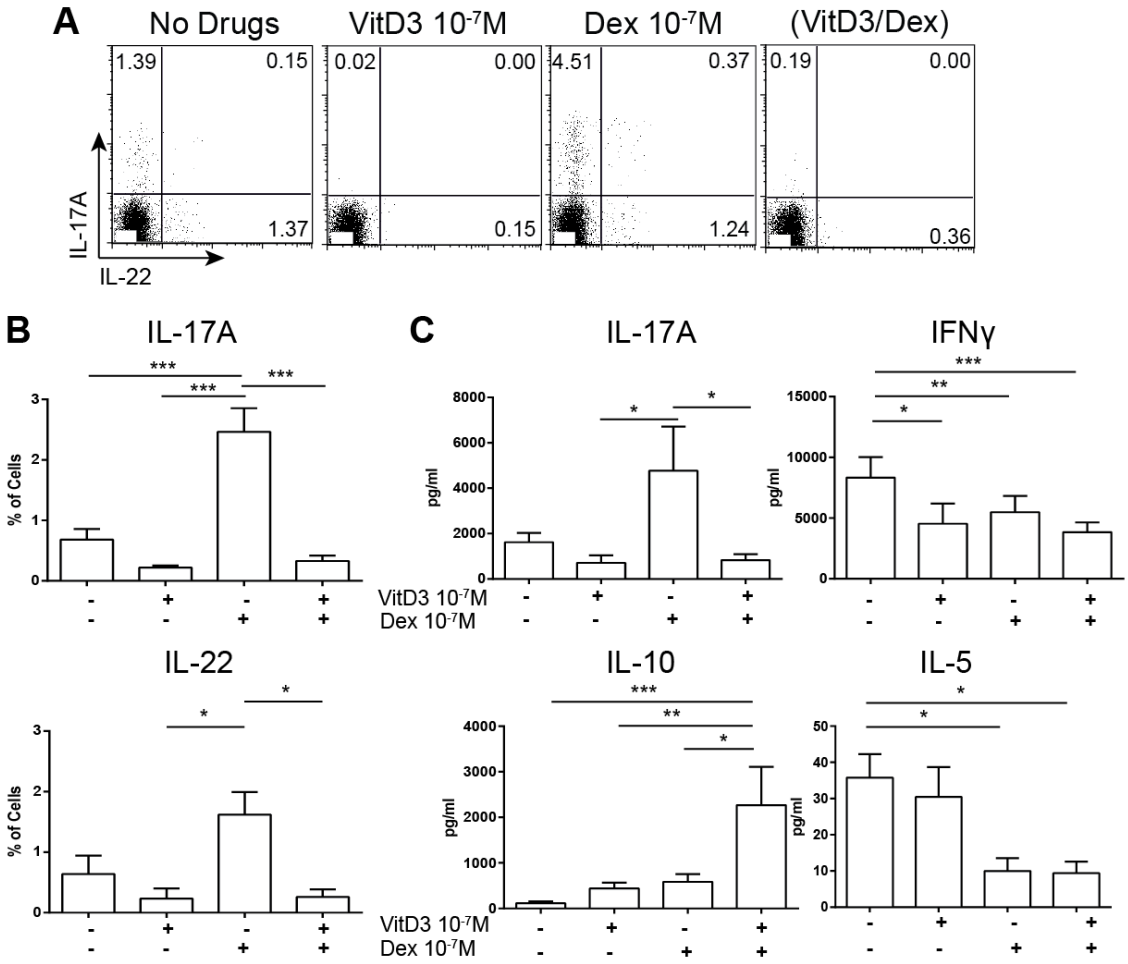


Figure 4-2 Dexamethasone enhances IL-17A conversely to 1,25(OH)$_2$D3

CD8-depleted PBMCs stimulated for 7-days with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)2D3 (VitD3; 10xM) and/or dexamethasone (Dex; 10xM), assessed for IL-17A and IL-22 production by intracellular flow cytometry and CBA. **A,** representative dot plots **B,** cumulative data of intracellular staining of cytokines IL-17A (n=5) and IL-22 (n=3) **C,** cumulative cytokine secretion data from CD8-depleted cells (n=7). **B, and C,** cytokine production assessed by repeated-measures one-way ANOVA with Tukey's multiple comparison post-test * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$.

Dexamethasone was used at 10^{-7}M as determined by extensive earlier studies in the lab; found that this concentration had the optimum induction of IL-17A and other cytokines [185,187,349]. There was a significant increase in the frequency of IL-17A+ cells in cultures containing 10^{-7}M dexamethasone and there is a similar trend observed

in IL-22+ cells (Figure 4-2A and B). When 10^{-7}M 1,25$(OH)_2$D3 is present in the cultures there was a significant decrease in the frequency of IL-17+ and IL-22+ cells as compared to dexamethasone. Inhibition of IL-17A by 1,25$(OH)_2$D3 occurred independently of whether dexamethasone was present or not. There was a trend towards increase in the amount of IL-17A secreted in culture in the presence of dexamethasone ($p = 0.12$) which was significantly decreased with the presence of 1,25$(OH)_2$D3. The drugs dexamethasone and 1,25$(OH)_2$D3 still had the predicted effects on Th1 and Th2-associated cytokines with a reduction in IFNγ and IL-5, whilst significantly increasing IL-10 production when both drugs were present (Figure 4-2C).

4.2.3 Dexamethsone significantly inhibits proliferation, whereas 1,25$(OH)_2$D3 does not in culture.

As asthma is proposed to predominantly be a CD4+ T cell-mediated disease, and it is predicted that the majority of the Th17-associated cytokines come from CD4+ T cells both CD8-depeleted PBMCs and CD4+ T cells were investigated. To determine if the inhibition of IL-17A by 1,25$(OH)_2$D3 was due to inhibition of proliferation, CD8-depleted PBMCs and CD4+ T cells were labeled at Day 0 with CellTrace Violet and cultured for 7-days with anti-CD3 and IL-2. Intracellular cytokine production and proliferation were assessed by flow cytometry on Day 7.

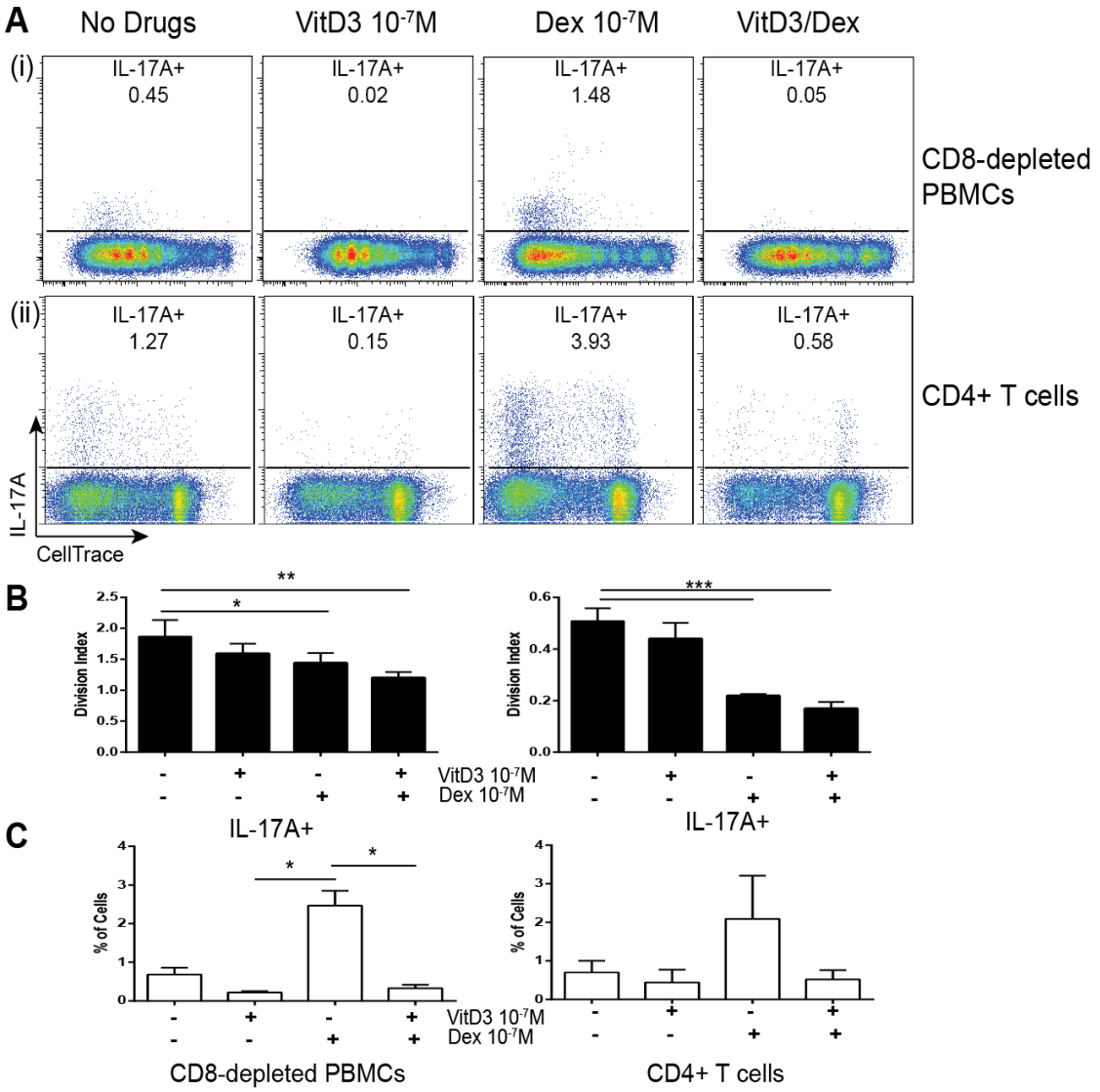


Figure 4-3 Dexamethasone inhibits proliferation whereas 1,25(OH)$_2$D3 does not

CD8-depleted PBMCs or CD4+ T Cells stimulated for 7-days with anti-CD3 and IL-2 (No drugs; -) or additionally with 1,25(OH)$_2$D3 (VitD3; 10^{-7}M) and/or dexamethasone (Dex; 10^{-7}M). Intracellular cytokine expression was assessed at Day 7 post 4 hour incubation with PMA and Ionomycin with the final 2hours containing Monensin. Proliferation was assessed by loss of fluorescence of CellTrace violet at Day 7. **A,** representative histograms of **(i)** CD8-depleted PBMCs and **(ii)** CD4+ T cells **B,** Cumulative data of division index of CD8-depleted PBMCs (n=4; left) and CD4+ T cells (n=5; right) **C,** Cumulative data of intracellular IL-17A staining of CD8-depleted PBMCs (n=4; left) and CD4+ T cells (n=3; right). **B, and C,** proliferation and % positive cells respectively assessed by repeated-measures one-way ANOVA with Tukey's multiple comparison post-test * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$.

Dexamethasone significantly inhibited proliferation of CD8-depleted and CD4+ T cells independent of whether 1,25$(OH)_2$D3 was present or not. However, in both CD8-depleted and CD4+ cell cultures the majority of IL-17A is being produced by cells that had proliferated (Figure 4-3).

4.2.4 Comparison of CD39 and CD73 expression *ex vivo* in severe asthma and healthy control peripheral blood.

CD39 is an ecotnucleotidase expressed on the cell surface of Foxp3+ Tregs and memory CD4+ T cells [136,418,419]; it breaks down proinflammatory extracellular ATP to AMP. This generated AMP is rapidly converted by CD73, to the immunomodulatory molecule Adenosine [137,139,418], as can be seen below in Figure 4-2:

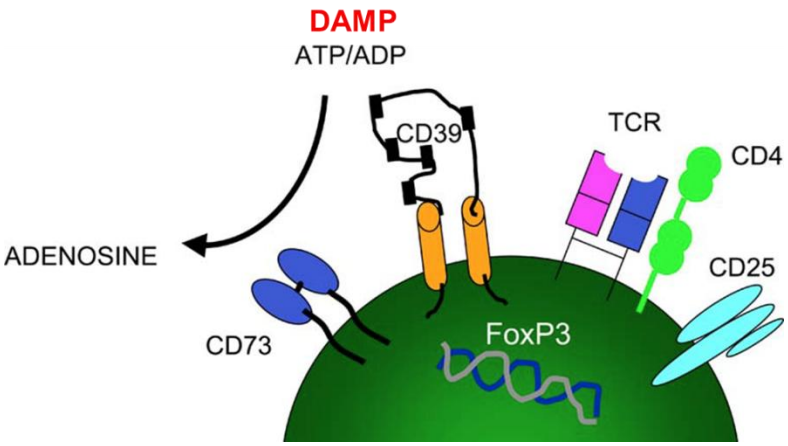


Figure 4-4 Schematic of the CD39/CD73 pathway.

Extracellular ATP acts as a Danger associated molecular pattern (DAMP), however ATP is broken down into AMP by CD39 and this is further catalysed to adenosine by CD73. Taken from [420]

What is becoming increasingly clear is that CD39+Foxp3+ T cells play an important role in ameliorating Th17-mediated disease, as it was shown that only CD39+Foxp3+ T cells and not CD39-Foxp3+ T cells could control Th17 cytokine production *in vitro* [138].

Multiple Sclerosis (MS) is an autoinflammatory condition assumed to be driven at least in part by an inappropriate Th17 immune response. MS patients were assessed and found to have reduced frequency and function of CD39+Foxp3+ CD4+ T cells [138]. This has subsequently been confirmed by other groups showing that CD39+ T cells are important for controlling Th17 cells [140,418].

The severe asthma patients assessed had significantly higher levels of IL-17A and IL-22 in culture, therefore as CD39 has been implicated as being important for controlling Th17 cells the frequency of CD39 and CD73 cells were investigated *ex vivo*. Peripheral blood of severe asthmatics and healthy controls were stained for CD39, CD73, CD3, CD25 and Foxp3, and the gating strategy to assess CD39+ and CD73+ populations can be found below in Figure 4-5.

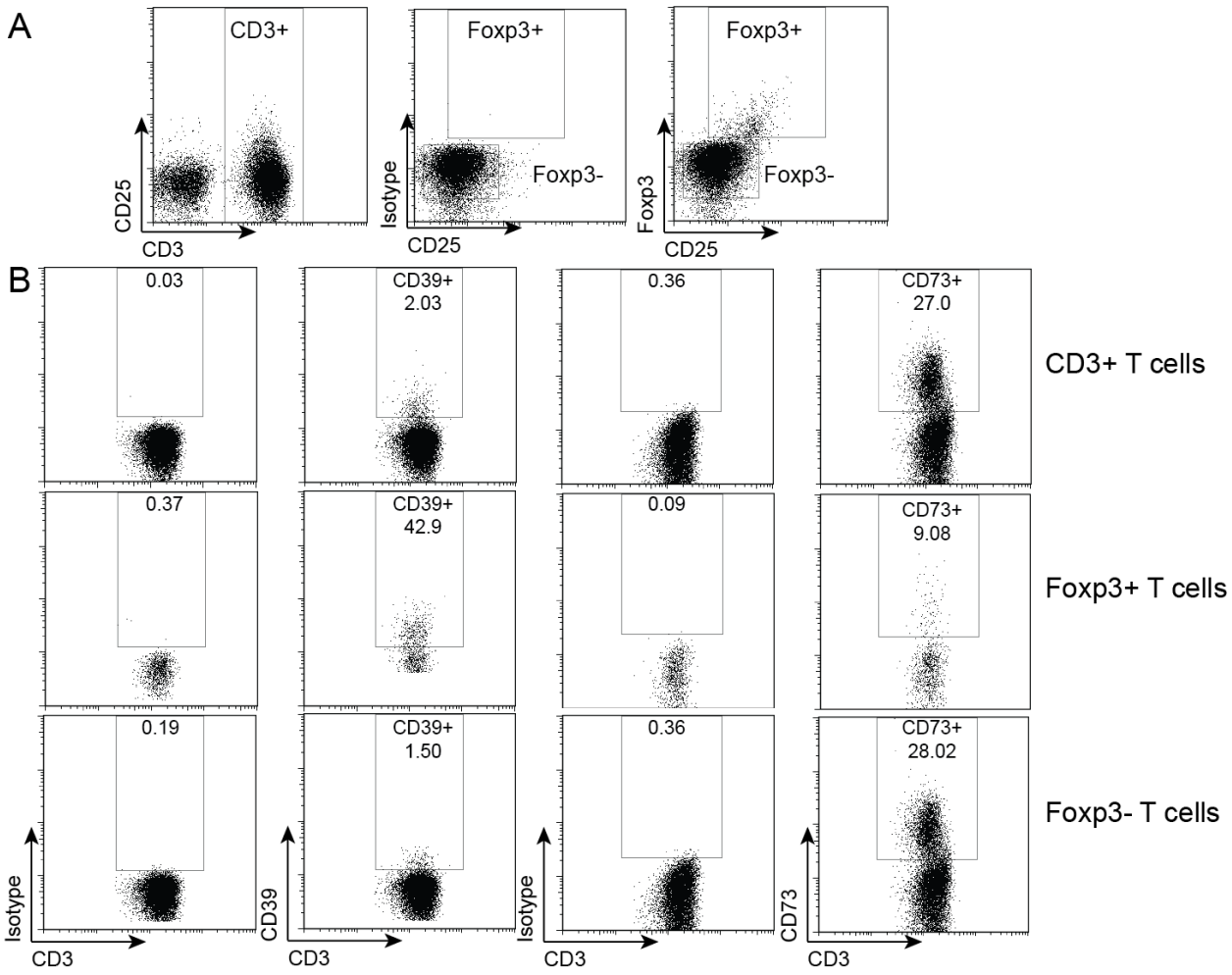


Figure 4-5 Representative staining showing the gating strategy for CD39 and CD73 *ex vivo* analysis

Direct *ex vivo* flow cytometric analysis of whole peripheral blood obtained from a representative healthy donor **A,** gating strategy for CD3+, then within the CD3+ gate Foxp3+ and Foxp3- gates based on isotype control staining **B,** representative staining of CD39 (left) and CD73 (right) in CD3+ T cells (top), Foxp3+CD3+ T cells (middle) and Foxp3-CD3+ T cells (bottom).

CD39 and CD73 expression was assessed on all CD3+ T cells as well as Foxp3+ CD3+T cells and Foxp3-CD3+T cells (Figure 4-5). The cumulative results can be found below (Figure 4-6):

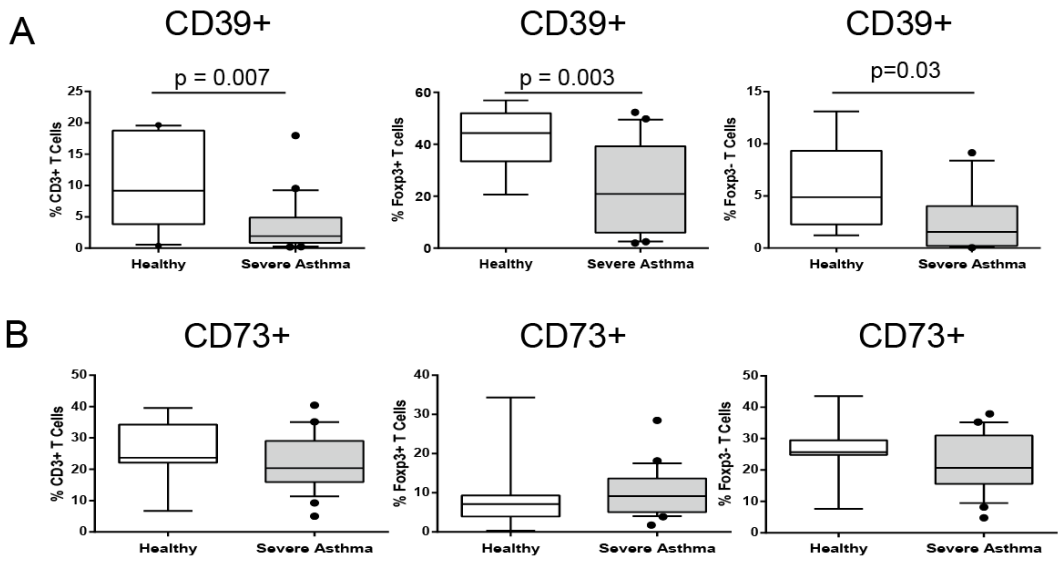


Figure 4-6 Increased frequency of CD39+ T cells in the peripheral blood of healthy donors as compared to severe asthmatics

Direct ex vivo flow cytometric analysis of peripheral blood obtained from healthy and severe asthma donors **A,** cumulative data of CD39 expression on CD3+ T cells (left), Foxp3+ T cells (middle) and Foxp3- T cells (right) in healthy (n=8) and severe asthma (n=23) **B,** cumulative data of CD73 expression on CD3+ T cells (left), Foxp3+ T cells (middle) and), Foxp3- T cells (right) in healthy (n=8) and severe asthma (n=23) **A, and B** % of positive cells were in healthy versus severe asthmatics were assessed by unpaired t-test. Data shown for the severe asthmatics was from Screening Visit 1.

There was no significant difference in the frequency of CD39+ or CD73+ CD3+T cells ($p = 0.25$ and $p = 0.70$ respectively) in the peripheral blood of SS versus SR asthmatics (data not shown). However, when comparing healthy and severe asthmatics there was a significant increased frequency of CD39+CD3+ cells in healthy donors as compared to severe asthmatics. As CD39 has predominantly been associated with being expressed in Foxp3+ T cells, Foxp3 was costained with CD39. There were significantly more CD39+Foxp3+ T cells in the peripheral blood of healthy as compared to severe asthma donors. However, not all CD39+ cells were Foxp3+ and when Foxp3- CD3+CD39+ cells were assessed there was equally a significant difference between

healthy and severe asthmatics. There was no significant difference between the frequency of CD73+CD3+, CD73+Foxp3+ or CD73+Foxp3- cells in healthy and severe asthma.

As the severe asthma cohort had lower levels of serum 25(OH)D, the data was assessed to see if there was any correlation between CD39+ and CD73+ T cells and serum 25(OH)D.

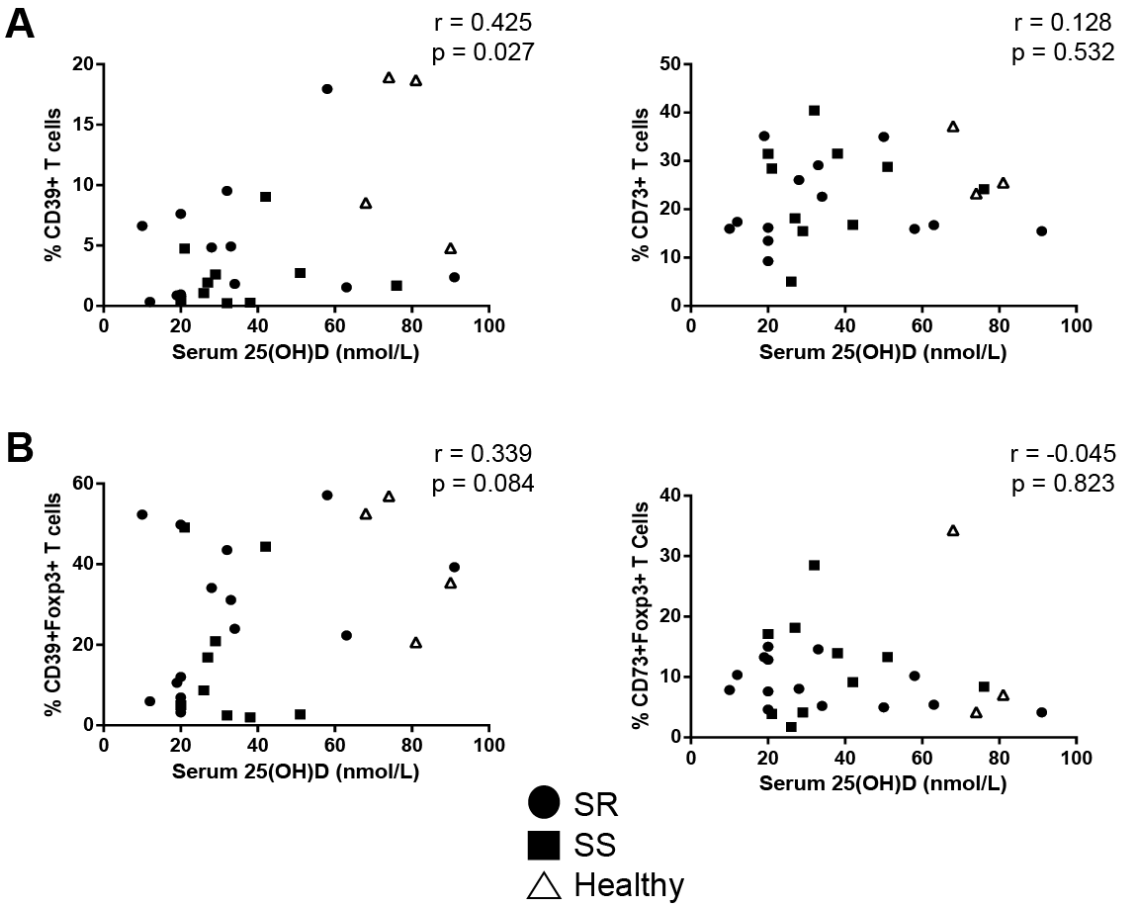


Figure 4-7 Serum 25(OH)D moderately correlated with CD39+ but not CD73+ T cell frequencies

Direct *ex vivo* flow cytometric analysis of peripheral blood obtained from healthy and severe asthma donors was carried out as described in Figure 4-5 **A,** correlation between serum 25(OH)D and CD39+ (right) and CD73+ (left) T cell frequencies **B,** correlation between serum 25(OH)D and CD39+Foxp3+ (right) and CD73+Foxp3+ (left) T cell frequencies. **A, and B** correlations between serum 25(OH)D and frequency of cells was assessed by Pearsons correlation test. SR closed circles, SS closed squares and Healthy open triangles. Data shown for the severe asthmatics was from Screening Visit 1

There was a significant moderate positive correlation between the frequency of CD39+ T cells and serum 25(OH)D (r = 0.42; p = 0.027) (Figure 4-7A). Also there was a trend towards a moderate positive correlation between the frequency of Foxp3+CD3+ T cells and serum 25(OH)D (r = 0.339; p = 0.084). There was no correlation observed between the frequency of CD73+ and CD73+Foxp3+ T cells and serum 25(OH)D (Figure 4-7A and B).

4.2.5 1,25(OH)$_2$D3 increases CD39 and CD73 expression on CD4+ T cells in vitro

As 1,25(OH)$_2$D3 has the capacity to inhibit IL-17A production as well as there being a moderate positive correlation between frequency of CD39+ T cells and serum 25(OH)D3, it was next investigated if 1,25(OH)$_2$D3 had any effect on CD39 and CD73 expression on CD4+ T cells in culture. CD4+ T cells were isolated by positive selection from the peripheral blood of healthy donors, and cultured for 7-days with anti-CD3 and IL-2 in the presence or absence of drugs as indicated. The effects of 1,25(OH)$_2$D3 on CD39 and CD73 expression *in vitro* was assessed by investigating gene and protein expression.

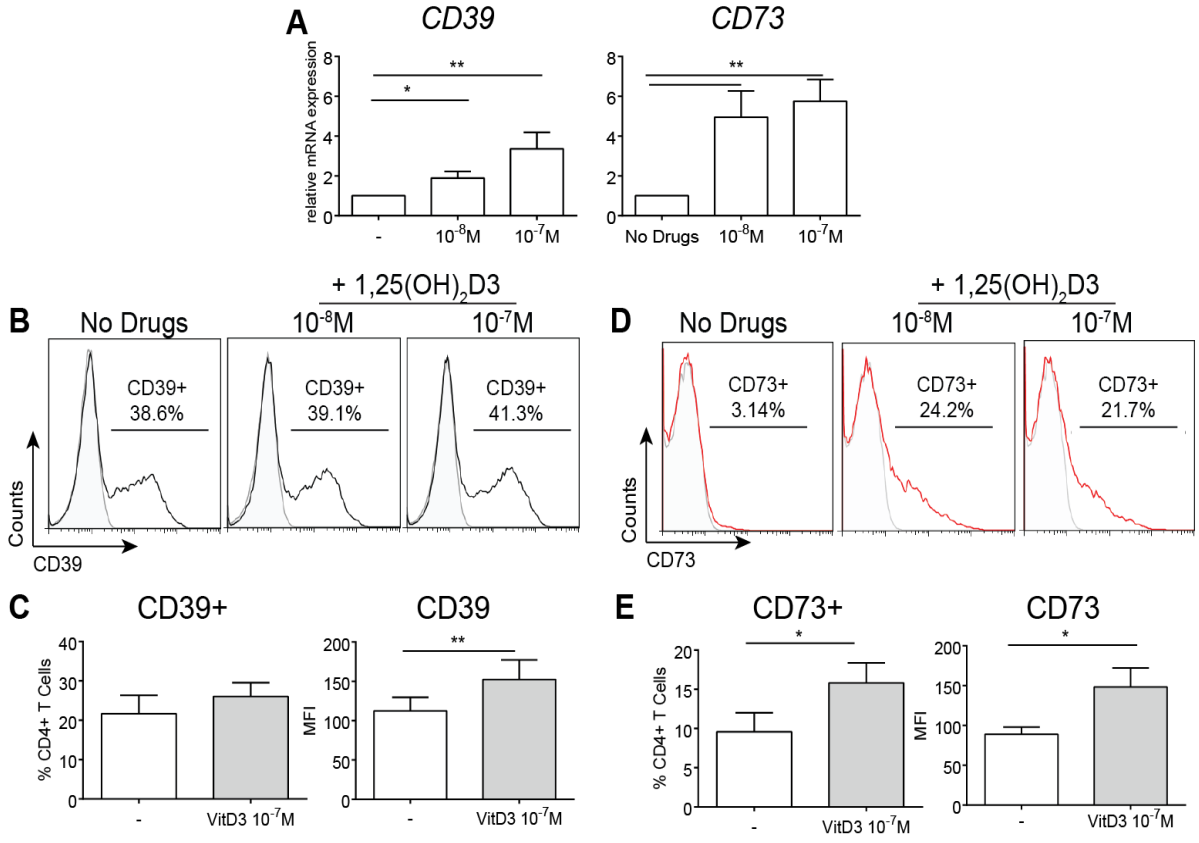


Figure 4-8 1,25(OH)$_2$D3 increased the frequency and expression of CD39 and CD73 on healthy CD4+ T cells *in vitro.*

CD4+ T Cells stimulated for 7-days with anti-CD3 and IL-2 (No drugs**; -**) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{X}M) **A,** cumulative data of relative mRNA expression of CD39 and CD73 gene expression (n=7) **B,** representative histograms based on isotype control (grey) and **C,** Cumulative data of cell surface expression of CD39 (n=6) (% of cells (left); MFI of positive cells (right)) **D,** representative histograms based on isotype control (grey) and **E,** Cumulative data of cell surface expression of CD73 (n=6) (% of cells (left); MFI of positive cells (right). **A,** comparison between relative mRNA expression assessed by Freidman test with Dunn's multiple comparison post-test and **C, and E,** differences in frequencies of cells cultured with or without 1,25(OH)$_2$D3 was assessed by paired t-test. * $p \leq 0.05$, ** $p \leq 0.01$.

1,25(OH)$_2$D3 significantly increased the gene expression of CD39 (Ectonucleoside triphosphate diphosphohydrolase 1 – gene name) and CD73 (5'-nucleotidase, ecto – gene name) in CD4+ T cells cultured *in vitro* for 7 days as compared to the no drug

condition (Figure 4-8A). To assess whether a similar pattern was seen at the protein level, cell surface flow cytometry was performed. There was a trend for 1,25$(OH)_2$D3 to increase the frequency of CD39+ CD4+ T cells (p=0.19), however there was a significant increase in the expression of CD39 on the CD39+ cells as determined by MFI expression (Figure 4-8B and C). 1,25$(OH)_2$D3 also significantly increased the frequency CD73+ CD4+ T cells as compared to no drug conditions. In conjunction with this 1,25$(OH)_2$D3 also significantly increased the expression of CD73 on CD73+ cells (Figure 4-8D and E).

To further phenotype 1,25$(OH)_2$D3-induced CD39+ and CD73+ CD4+ T cells, costaining was performed with intranuclear Foxp3 staining, as CD39 and CD73 have predominantly been associated with being Foxp3+ Treg markers.

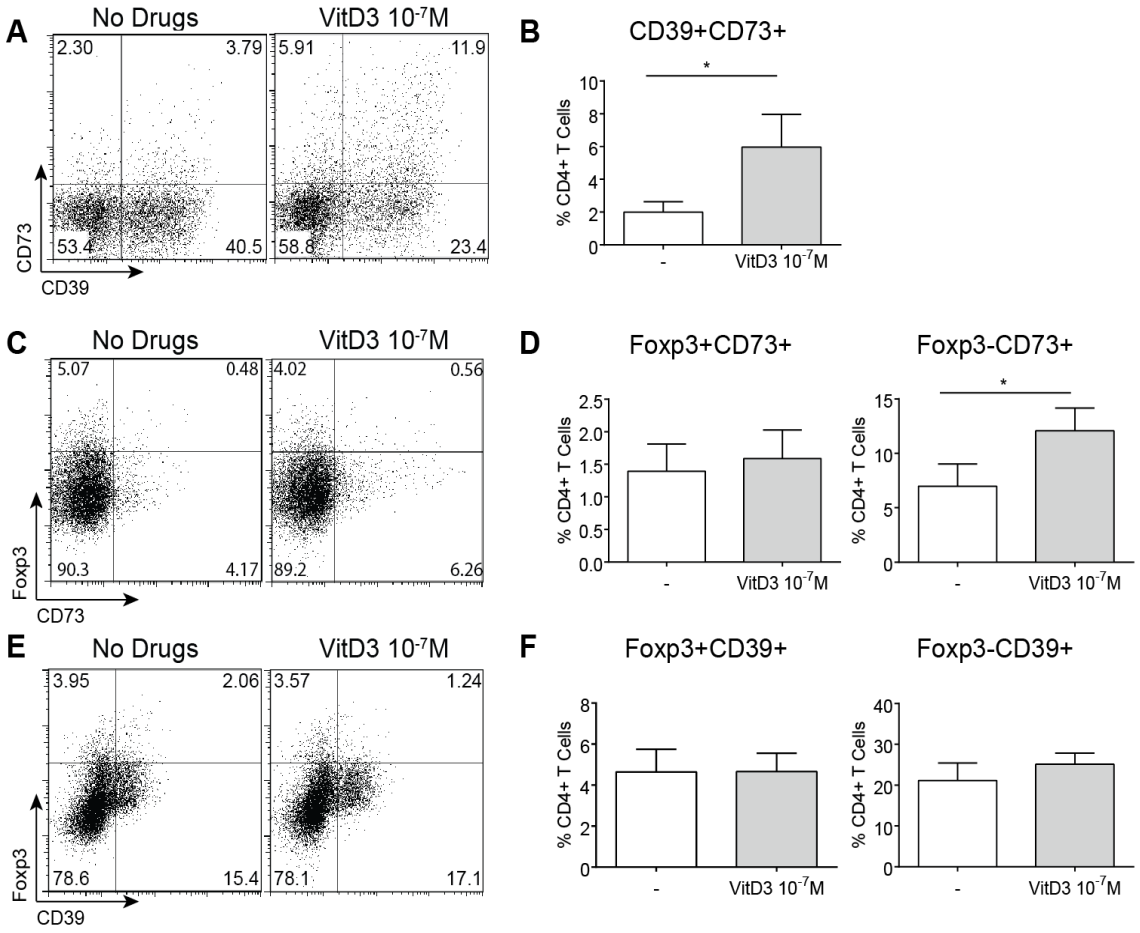


Figure 4-9 1,25(OH)$_2$D3 increased the frequency and expression of CD39+CD73+ but not CD39+Foxp3+ or CD73+ Foxp3+ CD4+ T cells in vitro.

CD4+ T Cells stimulated for 7-days with anti-CD3 and IL-2 (No drugs; -) or additionally with 1,25(OH)$_2$D3 (VitD3; 10^{-7}M) **A,** representative dot plots **B,** cumulative data of the frequency of CD39+CD73+ CD4+ T cells (n=6) **C,** representative dot plots **D,** cumulative data of the frequency of CD73+Foxp3+ (left) and CD73+Foxp3- CD4+ T cells (n=6) **E,** representative dot plots **F,** cumulative data of the frequency of CD39+Foxp3+ (left) and CD39+Foxp3- CD4+ T cells (n=6) **B, and D, and F,** differences in the frequency of Foxp3+ CD4+ T cells when cultured with or without 1,25(OH)$_2$D3 was assessed by paired t-test. * $p \leq 0.05$, ** $p \leq 0.01$.

1,25(OH)$_2$D3 significantly increased the co-expression of CD39 and CD73 on CD4+ T cells as compared to the no drug condition (Figure 4-9A and B). There was no significant difference in the frequency of CD73+Foxp3+ or CD39+Foxp3+ cells cultured

with or without 1,25$(OH)_2$D3. However, 1,25$(OH)_2$D3 significantly increased the frequency of CD73+ on Foxp3- cells (Figures 4-9C-F).

4.2.6 Inhibition of CD39 enzymatic activity with POM-1

To assess the role of 1,25$(OH)_2$D3-induced CD39 in inhibition of IL-17A production, a CD39 inhibitor was used, POM-1, which inhibits the enzymatic activity of CD39. As the original observations about 1,25$(OH)_2$D3 inhibition of IL-17A production were observed in CD8-depleted PBMCs, CD8-depleted PBMCs were cultured in the presence or absence of 1,25$(OH)_2$D3 and POM-1. Cells were recounted at Day 7 and re-cultured at $1x10^6$/ml in the presence of anti-CD3 and IL-2 in the absence of drugs for a further 48-hours when supernatants were collected and cytokine production was assessed by Cytometric Bead Array (CBA).

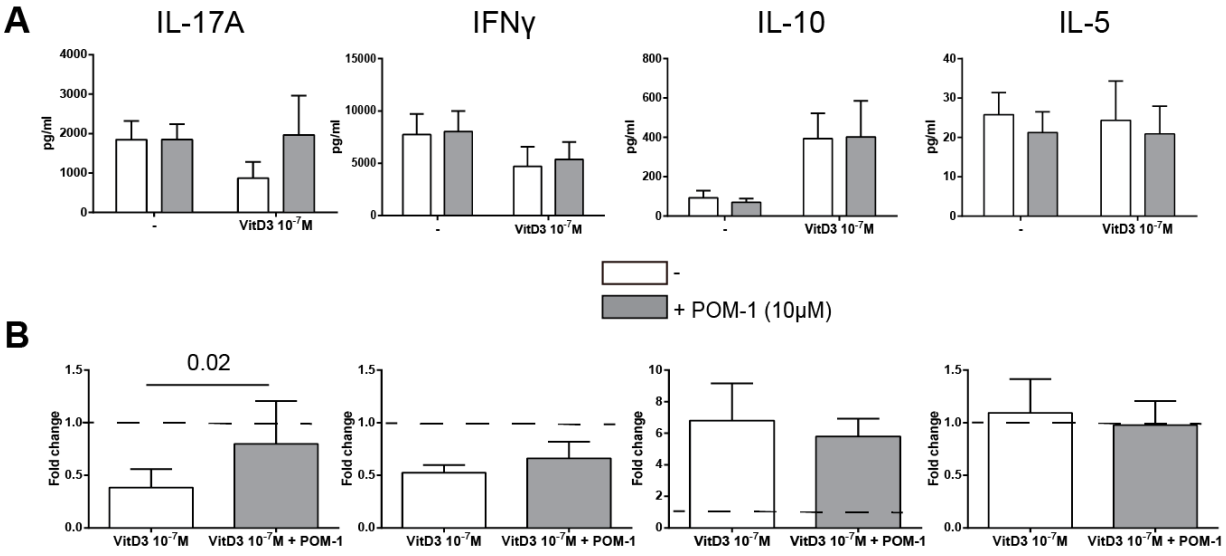


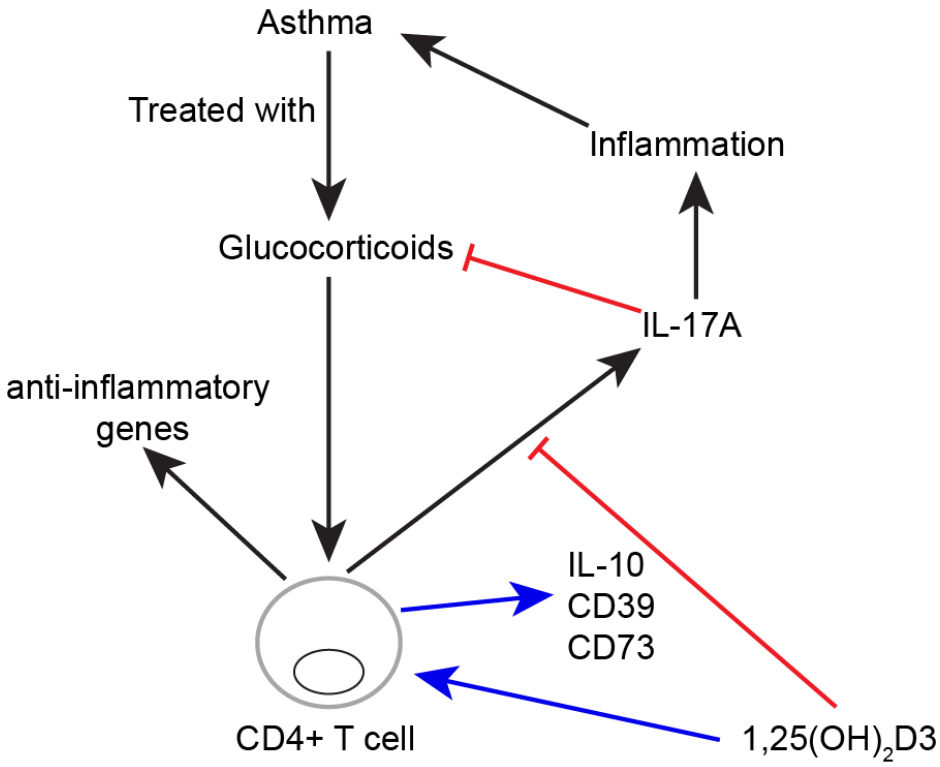
Figure 4-10 Inhibition of CD39 by POM-1 partially abrogates the inhibition of IL-17A by 1,25(OH)$_2$D3

CD8-depleted PBMCs were cultured for 7-days with anti-CD3 and IL-2 (No drugs) or additionally with 1,25(OH)$_2$D3 (VitD3; 10^{-7}M) and/or POM-1 (10μM) **A,** cumulative data of cytokine production as measured by CBA (n=7) **B,** cumulative cytokine data normalised to the no drug condition (1 = no drug level of cytokine production) (n=7). **B,** differences in cytokine production assessed by paired t-test.

As expected when cells were cultured in the presence of 1,25(OH)$_2$D3 there was enhancement of IL-10 production and inhibition of IFNγ and IL-17A. The addition of POM-1 into culture had no significant effect on IL-10, IL-5 or IFNγ cytokine production in the presence or absence of 1,25(OH)$_2$D3 (Figure 4-10A). However the addition of POM-1 to 1,25(OH)$_2$D3 cultures enhanced IL-17A production, and nearly restored it back to no drugs levels. Due to the large donor variation this did not reach significance, however when the data was analysed as a ratio to the no drug condition as seen in Figure 4-10B, there was a significant increase in IL-17A when POM-1 was added to 1,25(OH)$_2$D3 cultures. There was no significant difference seen with IL-10, IL-5 or IFNγ (Figure 4-10A and B).

4.3 Discussion

The data in this chapter demonstrates that the production of IL-17A and IL-22 by human peripheral blood cells is elevated in severe asthma. The glucocorticoid dexamethasone enhanced the frequency of IL-17A positive cells and IL-17A production in non-asthmatic control cultures. In contrast the active form of vitamin D reduced both cytokines directly and also when dexamethasone was present in culture. $1,25(OH)_2D3$ increased expression of CD39 and CD73, and also significantly increased CD39+CD73+ CD4+ T cells. However unexpectedly the population of CD39+ and CD73+ CD4+ T cells induced by $1,25(OH)_2D3$ are not Foxp3+ but Foxp3- T cells. Together with data using the CD39 inhibitor POM-1, this work suggests a role for the ectonucleotidase CD39 in $1,25(OH)_2D3$ mediated inhibition of the IL-17A response. This work leads us to propose the following model:



4-11 Schematic representation of our proposed role for glucocorticoids and $1,25(OH)_2D3$ in IL-17A-mediated severe asthma

Asthma is treated with glucocorticoids, and although they have broad anti-inflmamatory properties, we propose that they also enhance production of IL-17A. IL-17A has been previously linked with increased steroid resistance as well as increasing inflammation and enhancing the asthmatic disease. We propose that $1,25(OH)_2D3$ inhibits IL-17A production from CD4+ T cells through increase in IL-10 production as well as increase in surface expression of CD39 and CD73.

This work extends earlier observations from a number of groups showing an association between elevated IL-17A production and severe asthma [370,371]. However this study was novel as the severe asthmatics were further characterised as being SS and SR, based on their clinical response to steroids. While cells from all asthmatics demonstrated elevated IL-17A and IL-22 synthesis, the most striking difference was in the levels of secreted IL-17A where there was 7-fold more IL-17A produced in the SR as compared to SS. This data is the first human study to highlight

the higher expression of IL-17A in clinically-defined SR asthmatics. It extends earlier findings that showed that Th17- rather than Th2-mediated inflammation in an allergic mouse model of asthma was not inhibited by steroids [404]. In contrast there was no difference in levels of IL-22 produced between SS versus SR. This is interesting as IL-22 and IL-17A are both proposed to be expressed by Th17 cells, and earlier data suggests that IL-22 enhances IL-17A inflammation, thus differences in IL-17A are more important with regards to Th17-driven inflammation then IL-22 [56]. In OVA-driven allergic mouse models of asthma it was identified that high expression of IL-22 can prevent allergic inflammation [55,410]. So what is really interesting is that there were a significantly higher proportion of IL-17A+ IL-22+ double positive cells in severe asthmatics as compared to healthy non-asthmatic controls. These data support earlier reports highlighting that double positive conventional T cells are proposed to represent the more pathogenic population with studies showing that as both IL-17A and IL-22 enhances the pro-inflammatory properties of each other [56].

There is a single study suggesting that IL-17A initiates disease, however that it also plays a role in controlling established allergen-driven inflammation [421]. However whether these experimental models accurately reflect the complex disease that is human SR asthma, particularly airway remodelling a defining characteristic of asthma is unclear. The majority of publications provide evidence that Th17 cytokines play a mechanistic role in increasing asthma severity and reducing corticosteroid sensitivity [405,406] and a number of human studies have shown association between IL-17A levels with asthma severity [370,371,407]. Nevertheless, we cannot completely exclude the possibility that elevated IL-17A production by severe asthma is associated with inhaled corticosteroid administration since all of the severe asthma patients recruited were on high-dose of inhaled steroids for the duration of the study (Table 3-1), and we observed that a positive correlation exists between the dose of inhaled steroids and

amounts of blood PBMC IL-17A released in vitro (Pearson correlation r=0.459; p=0.014) (Alexandra Nanzer and ESC, unpublished observations).

Dexamethasone significantly increased the frequency of IL-17A+ cells and IL-17A secretion in cells isolated from the peripheral blood of healthy subjects. Conversely $1,25(OH)_2D3$ significantly inhibited IL-17A production in a dexamethasone-independent mechanism. Additional issues with high IL-17A is the reported upregulation of GRβ, which is an inhibitory receptor for GRα (the functional receptor) for glucocorticoids and this may worsen disease and also prevent clinical response to treatment [360,409]. These data imply a complex and potentially detrimental relationship between IL-17A, steroid treatment and responsiveness in severe asthma, on which vitamin D may have a positive impact. It is interesting to note that we and others have shown that both $1,25(OH)_2D3$ and dexamethasone increase the expression of the anti-inflammatory cytokine IL-10 *in vitro*, and IL-10 has been shown to inhibit IL-17A production [186,347,349,350,422-424]. So it is interesting that only $1,25(OH)_2D3$ inhibits IL-17A production, and this could be due to additional mechanisms shown in this report such as CD39 or due to differential effects on Th1- and Th2-associated cytokine production. Dexamethasone was shown to be more inhibitory on Th2-associated cytokine production then $1,25(OH)_2D3$, and this is good in the context of Th2-associated disease associated with milder and allergic forms of asthma. However Th2-associated cytokines have also been shown to play an important role in inhibiting IL-17A cytokine production [422,425]. Thus blocking Th2 responses by dexamethasone may allow the Th17 response to develop. This could represent a potential mechanism through which dexamethasone is less inhibitory on Th17 cytokines, and perhaps suggests a contributory mechanism through which steroids may enhance Th17 disease.

With hindsight it would have been informative to have included IL-17F in the staining and CBA protocol as the cytokine IL-17F has been associated with IL-17A production as well as severe asthma [371,426]. IL17F and other Th17-associated cytokines can

also be assessed in stored sera from these patients in the future. Also it would be very informative to investigate the expression of GRα/β in our severe asthma cohort and compare this to healthy controls to determine if there is any correlation with cytokine response.

As CD39+Foxp3+ T cells have been implicated in controlling Th17-mediated disease [138,140], CD39 and its associated marker CD73 were investigated directly *ex vivo* by flow cytometric analysis. What was interesting was that there was a significantly increased frequency of CD39+ T cells in the peripheral blood of healthy donors as compared to severe asthmatics. When this was further analysed based on Foxp3 expression, there was significantly higher frequency of Foxp3+CD39+ T cells as well Foxp3-CD39+ T cells in the peripheral blood of healthy controls as compared to severe asthmatics. There was no significant difference in the frequency of CD73+ T cells of the peripheral blood of healthy and severe asthmatics. This is perhaps not surprising as in the CD39-CD73 pathway converting ATP to Adenosine CD39 is considered the rate limiting step and actually the conversion of AMP to Adenosine by CD73 happens almost instantly [137]. Some Healthy donors were further characterised by cell surface staining to identify whether the CD3+ T cells were CD4+ or CD8+ T cells. It was found that CD39 was predominantly expressed on the surface of CD4+ T cells, whereas CD73 was expressed on CD8+ and CD4+ cells (data not shown).

As $1,25(OH)_2D3$ was efficient at inhibiting IL-17A production, modulation of CD39 and CD73 on CD4+ T cells was investigated. As expected activation in culture itself upregulated CD39 expression [427], however when the level of expression of CD39 was investigated further it was found that $1,25(OH)_2D3$ increased the expression of CD39 on the CD39+ T cells i.e. there was no difference in frequency but a significant increase in MFI values. Also $1,25(OH)_2D3$ significantly enhanced CD73 number and expression of cells. The modulation of CD73 on CD4+ T cells by $1,25(OH)_2D3$ *in vitro* was a surprise as *in vivo* there was no correlation between serum 25(OH)D and CD73+

T cells, however it does suggest that in a local microenvironment i.e. within the lung $1,25(OH)_2D3$ may have the capacity to modulate CD73 expression. This would require further investigation perhaps by analysing BAL levels of 25(OH)D, although this still represents a technical challenge, and CD73 expression on T cells where you predict there may be a correlation. There was also a significant increase in the frequency of CD39+CD73+ cells present in $1,25(OH)_2D3$ cultures. Whether these cells are the most regulatory is yet to be investigated. The CD39+ and CD73+ cells induced by $1,25(OH)_2D3$ are predominantly Foxp3-, as there is no significant difference in CD39+Foxp3+ or CD73+Foxp3+ cells in the presence or absence of $1,25(OH)_2D3$. This is somewhat surprising as the majority of reports have stated that CD39 and CD73 are Foxp3+ Treg markers. However, perhaps these cells induced by $1,25(OH)_2D3$ are a different population of Treg, especially as the concentration of $1,25(OH)_2D3$ used 10^{-7}M, is known to induce IL-10+ Tregs rather than Foxp3+ [342] (and work presented in the next chapter) . Future work on this project will involve performing suppression assays to determine if CD39+ or CD73+ T cells are suppressive of CD4+ T cell proliferation and also IL-17A production. It will also be interesting to measure the levels of ATP and Adenosine in the supernatants of the samples to assess the function of CD39 and CD73.

In the context of other Th17-mediated inflammatory diseases, expansion of CD39+Foxp3+ in patients with Multiple Sclerosis (MS) has been shown to correlate with remission [428]. Conversely a reduction in CD39+ Treg function correlates with disease progression, and certainly it has been shown that the expansion of CD39+Tregs is required for control of the Th17-mediated inflammation [136,138,428,429]. CD39 has been shown to be expressed on memory Th17 cells in a mouse model of transplantation [430]. CD73 has also recently been identified as a memory Th17 marker, in a study where peripheral blood CD73+CD4+ T cells were shown to correlate with active Inflammatory Bowel Disease, which is suggested to be a

predominantly Th17-mediated disease [431]. One could speculate that these CD39+ or CD73+ Th17 cells perhaps have a regulatory capacity, similar to what is seen in Th2-mediated inflammation where Foxp3+ Tregs need to co-express the Th2-specific transcription factor IRF4, to control Th2-mediated inflammation [95], suggesting that Tregs use components involved in T helper CD4+ cell differentiation pathway to control the respective inflammation [95].

To further elucidate the role of CD39 in $1,25(OH)_2D3$ cultures the inhibitor POM-1 was used in CD8-depleted cultures containing $1,25(OH)_2D3$ to inhibit CD39 function. POM-1 partially abrogated the inhibition of IL-17A responses by $1,25(OH)_2D3$, suggesting that CD39 plays a role in $1,25(OH)_2D3$-inhibition of IL-17A. It would have been interesting to observe if full restoration of IL-17A would have been observed if a combination of POM-1 and αIL-10R (IL-10R blocking antibody) was used, as it is already known Th17-cells express the IL-10R and IL-17A production is inhibited by IL-10 [422-424]. Also $1,25(OH)_2D3$ can strongly induce IL-10, my and earlier data from the lab show [342,347,350].

There are many ways in which IL-17A may drive asthmatic disease including bronchial smooth muscle hypertrophy and hyperplasia, and subepithelial fibrosis [432]. We and others have previously demonstrated a negative association between 25(OH)D status and airways smooth muscle mass [373,433]. Certainly the asthma patients we assessed had very low levels of serum 25-hydroxyvitamin D (25(OH)D) (as shown in Figure 3-14), and this may have reduced their responsiveness to corticosteroids and enhanced their IL-17A production [372,394]. Manipulation of vitamin D status for therapeutic benefit in asthma and other respiratory conditions is currently highly topical. Understanding the various mechanisms by which vitamin D controls respiratory health and steroid responsiveness is central in targeting this pathway therapeutically and these results provide support for an additional beneficial effect. We believe that evidence of $1,25(OH)_2D3$ down regulating pro-inflammatory cytokines such as IL-17A

and IL-22, its capacity to enhance anti-microbial pathways, regulatory T cells and other homeostatic mechanisms such as CD200 [434], are all likely to contribute to promoting homeostasis in the airway and lung health.

5. *In vitro* induction of $1,25(OH)_2D3$-induced T regulatory Cells: role of cytokine milieu.

5.1 Introduction

$1,25(OH)_2D3$ has been shown to increase Foxp3+ Treg frequency in several different experimental models. It was shown first in mouse models of autoimmune diseases that $1,25(OH)_2D3$ increases the frequency of CD4+CD25+ Treg populations [339,340,435]. Subsequently in humans $1,25(OH)_2D3$ has been shown to induce Foxp3+ Tregs from human CD4+ T cells *in vitro* in the presence of IL-2, and these Tregs express CTLA-4 [341]. It has also been shown that $1,25(OH)_2D3$ increases the suppressive capacity and function of human Foxp3+Tregs in culture [436].$1,25(OH)_2D3$ is also reported to indirectly enhance Foxp3+Treg populations by preventing maturation of DCs and upregulating inhibitory receptors such as ILT3 [334-337].

We have previously reported that $1,25(OH)_2D3$ enhances IL-10+ Tregs *in vitro* and *in vivo* [350,437]. Subsequent analysis demonstrated that the induced cells do not co-express IL-10 and Foxp3, and that the concentration of $1,25(OH)_2D3$ is important in determining whether IL-10+ or Foxp3+ Tregs are induced (Figure 5-1).

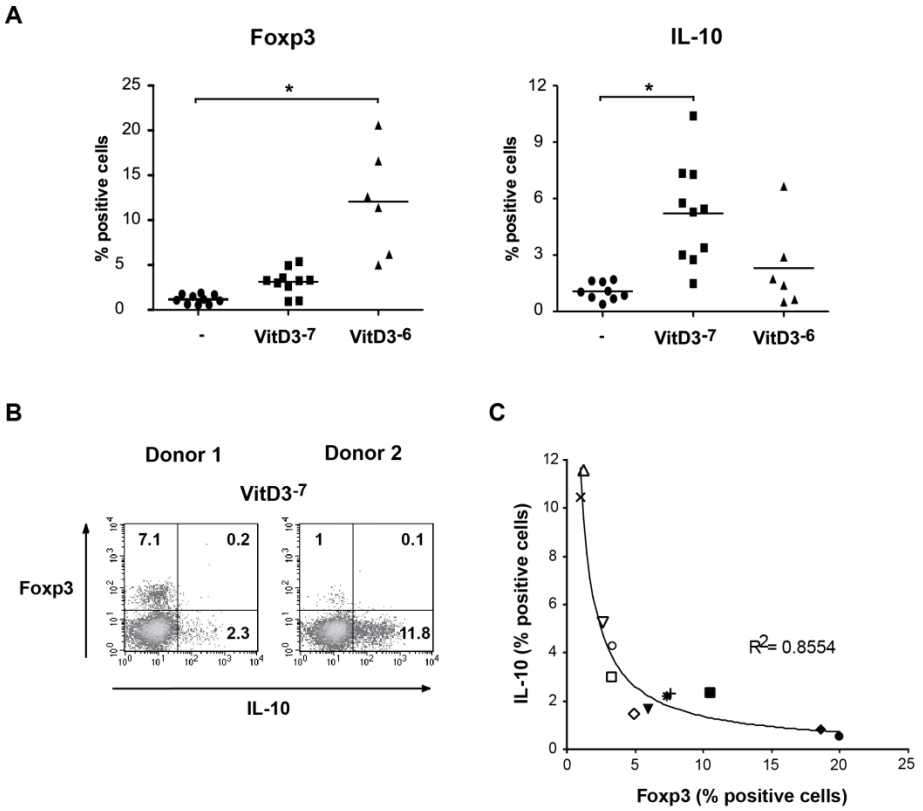


Figure 5-1 1,25(OH)$_2$D3 can increase the frequency of Foxp3+ and IL-10+ Treg populations.

Human CD4+ T cells were stimulated for two 7-day cycles with anti-CD3, IL-2 and IL-4 (No VitD3) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^xM). **A,** At day 14, cells were re-stimulated for 16 hours with anti-CD3 and IL-2. IL-10+ cells were identified using an IL-10 secretion assay kit. FoxP3+ cells were assessed by intranuclear staining. Values represent % of gated live CD4+ cells. Data from several (6-10 per group) healthy donors are depicted. **B,** Cells were co-stained for expression of both IL-10 and FoxP3 in the presence of 10^{-7}M 1,25(OH)$_2$D3. Two representative FACS plots from different donors are shown. Note the absence of FoxP3+IL-10+ cells. **C,** Data from the co-staining experiments depicted in a correlation analysis. Each symbol represents a different donors; closed symbols = 10^{-6}M 1,25(OH)$_2$D3, open symbols = 10^{-7}M 1,25(OH)$_2$D3. *=p<0.05, **=p<0.01 as determined by the Mann Whitney rank sum test. Taken from [342]

As can be seen from Figure 5-1 at the high doses of 1,25(OH)$_2$D3 (10^{-6}M) there is an increased frequency of Foxp3+ Tregs and no induction of IL-10+ Tregs, whereas

conversely at the lower doses of 1,25$(OH)_2$D3 (10^{-7}M) there is increased frequency of IL-10+ but not Foxp3+ Tregs. Also when co-staining was performed there was little if no co-expression of Foxp3 and IL-10, suggesting that these are distinct regulatory populations.

Further evidence suggesting a role for 1,25$(OH)_2$D3 in Foxp3+ Treg induction exists. We and others have demonstrated clear *in vivo* correlates showing that serum 25(OH)D levels correlate with Foxp3+ Tregs frequency and numbers in the periphery and in the airways (Chapter 3 Figure 3-15) [312,342,395]. Vitamin D status has also been linked to functionality of Tregs, as it was demonstrated in Multiple Sclerosis patients that low serum 25(OH)D status was associated with reduced suppressive capacity of $CD25^+CD4^+CD127^{lo}$ Tregs [396].

In this chapter I hypothesized that the cytokine milieu, as well as the concentration of 1,25$(OH)_2$D3, influences Foxp3+ Treg induction by 1,25$(OH)_2$D3. Hence the aim of this chapter is to build on earlier work from the lab, in particular to identify the potential mechanisms by which 1,25$(OH)_2$D3 at a high concentration (10^{-6}M) increases the frequencies of Foxp3+ Tregs, and also to identify the effects of cytokine milieu on Foxp3+ Treg induction by lower, potentially more physiologically relevant concentrations of 1,25$(OH)_2$D3.

5.2 Results

5.2.1 High dose 1,25(OH)$_2$D3 (10^{-6}M) exerts greater inhibition of Foxp3- T cell proliferation than Foxp3+ T cell proliferation

To build on previous observations from the lab showing that 1,25(OH)$_2$D3 at 10^{-6}M increases the frequency of Foxp3+ CD4+ T cells in culture, my work involved assessing potential mechanisms through which high dose 1,25(OH)$_2$D3 exerts its effect. In initial experiments proliferation of CD4+ T cells cultured in the presence or absence of different concentrations of 1,25(OH)$_2$D3, was assessed at Day 7 and Day 14. CD4+ T cells were labeled at Day 0 with CellTrace Violet and cultured for one or two rounds of 7-day culture with anti-CD3 and IL-2. Proliferation was assessed by loss of fluorescence of CellTrace.

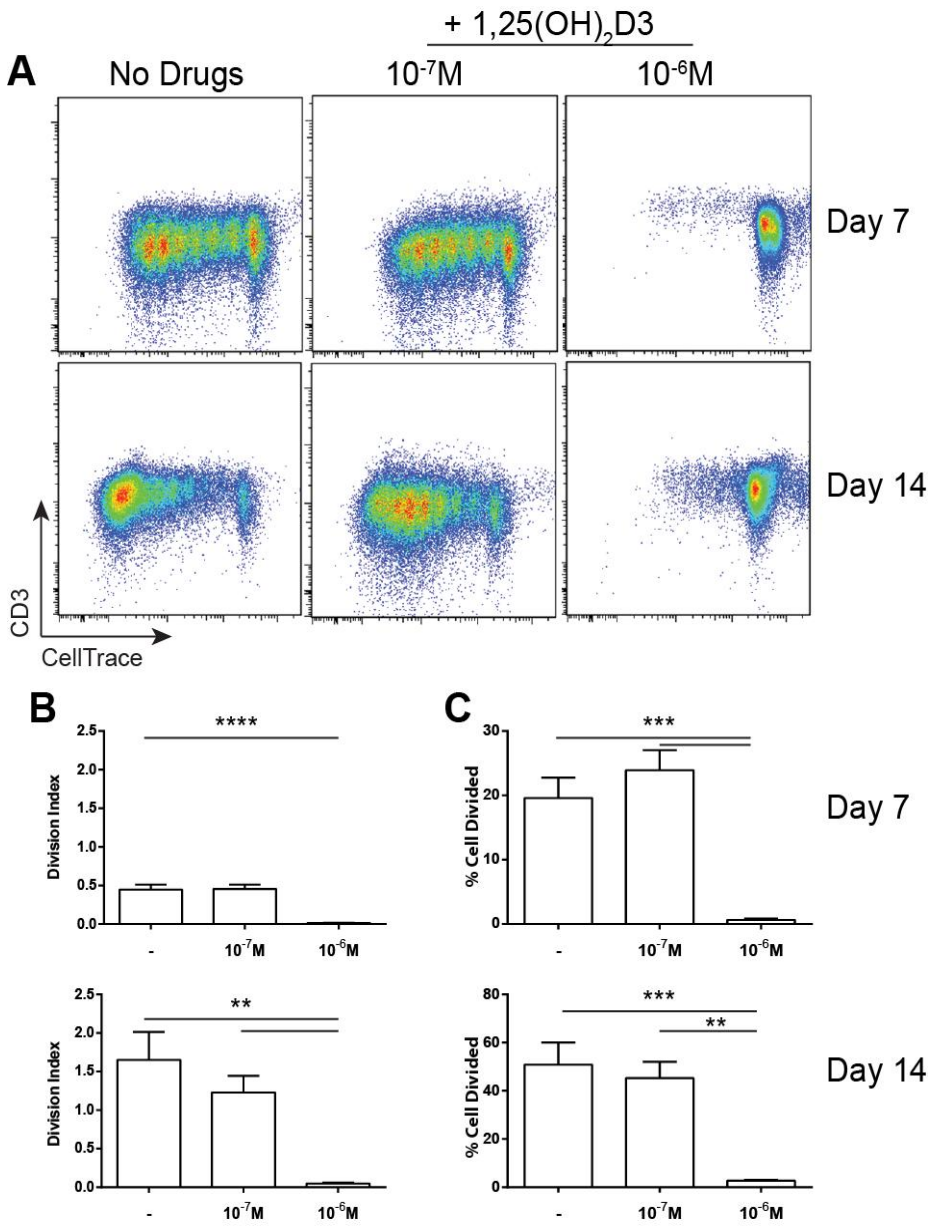


Figure 5-2 High dose 1,25(OH)$_2$D3 (10^{-6}M) inhibits proliferation of CD4+ T cells *in vitro*

CD4+ T Cells and then labeled at Day 0 with CellTrace Violet. Cells were stimulated for one or two 7-day cycles with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)2D3 (VitD3; 10-xM). Proliferation was assessed by loss in fluorescence. **A,** representative dot plots **B,** cumulative data of division index **C,** Cumulative data percent of original population divided after 7 days (top) and 14 days (bottom) **B, and C,** differences in proliferation assessed by a repeated-measures one-way ANOVA with Tukey's post-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

$1,25(OH)_2D3$ at 10^{-6}M significantly inhibited CD4+ T cell proliferation at Day 7 and Day 14. This was assessed in two ways, firstly by % of the original population of cells that had divided (Figure 5-2C) and secondly by Division index which is a ratio that calculates how many cells have undergone division (% divided) multiplied by the number of rounds of division (proliferation index) (Figure 5-2B).

To further elucidate the mechanism of 10^{-6}M $1,25(OH)_2D3$ effect on Foxp3+ T cells co-staining of CellTrace with Foxp3 was performed to investigate the proliferation of Foxp3+ versus Foxp3- cells. CD4+ T cells were labeled at Day 0 with CellTrace and cultured for 7-days or 14-days with anti-CD3 and IL-2 in the presence or absence of $1,25(OH)_2D3$ at the concentrations indicated, proliferation was assessed by loss of fluorescence of CellTrace. At Day 7 and Day 14 intranuclear Foxp3 staining was performed.

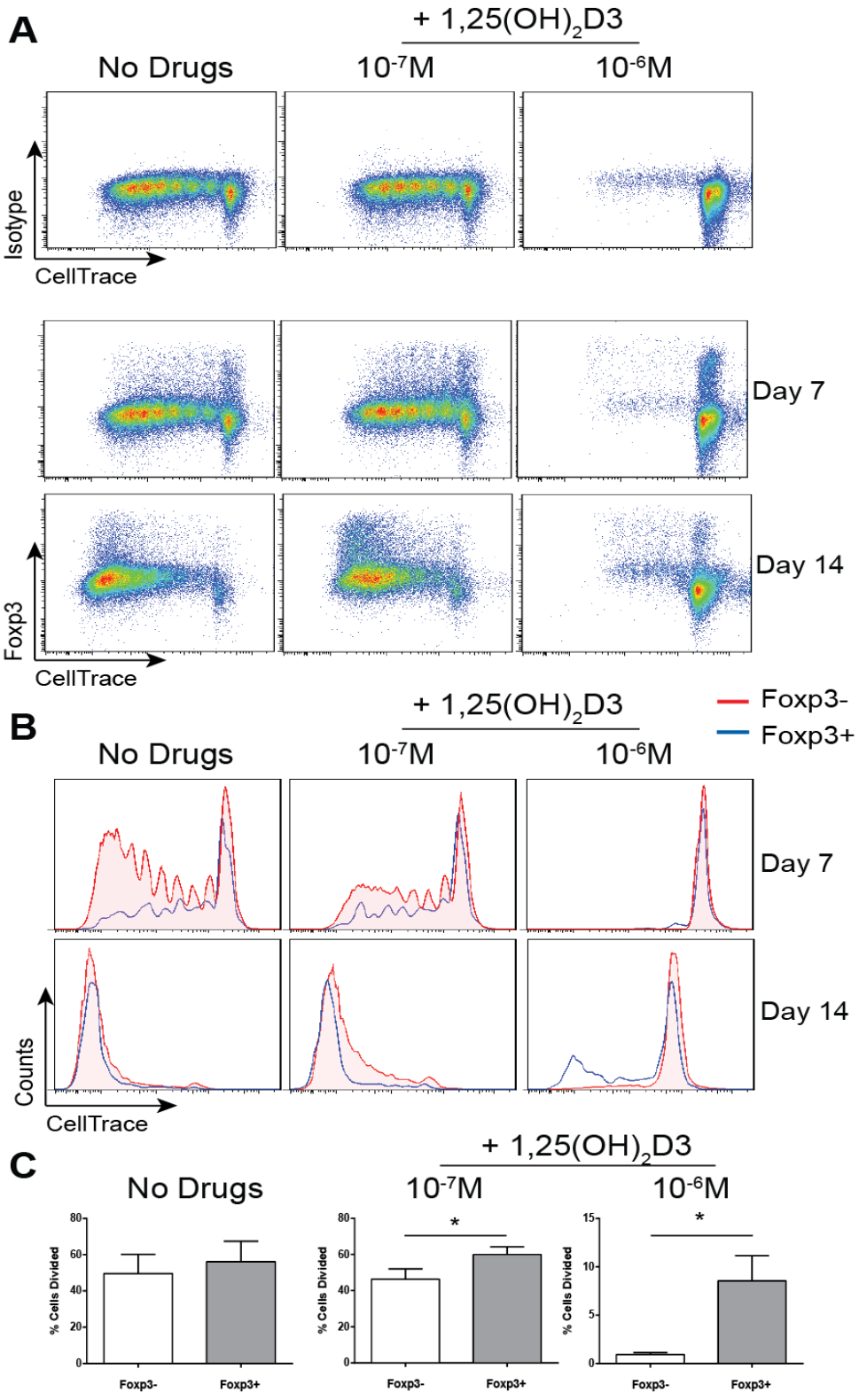


Figure 5-3 1,25(OH)$_2$D3 is less inhibitory on Foxp3+ as compared to Foxp3- CD4+ T cell proliferation

CD4+ T Cells stimulated for one or two 7-day cycles with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)2D3 (VitD3; 10-xM). Cells were labeled at Day 0 with CellTrace Violet and proliferation was assessed by loss in fluorescence **A,** representative dot plots **B,** representative histograms of Foxp3- (red) versus Foxp3+ (blue) proliferation with or without 1,25(OH)D3 after 7 days (top) or 14 days (bottom) of culture **C,** cumulative data of division index of Foxp3- (white) versus Foxp3+ (grey). **C,** differences in Foxp3+ and Foxp3- proliferation under different culture conditions were assessed by a paired t-test. * $p \leq 0.05$

As previously seen in Figure 5-2, 10^{-6}M $1,25(OH)_2D3$ inhibited proliferation of CD4+ T cells at Day 7 and Day 14 as compared to other conditions. Cells were gated based on whether they were Foxp3+ or Foxp3-. There was significantly less inhibition of Foxp3+ proliferation as compared to Foxp3- CD4+ T cells when 10^{-6}M $1,25(OH)_2D3$ was present (Figure 5-3 B and C). Also 10^{-7}M $1,25(OH)_2D3$ was significantly less inhibitory on Foxp3+ as compared to Foxp3- CD4+ T cell proliferation. There was no significant difference in Foxp3+ versus Foxp3- CD4+ T cell proliferation in the no drug condition.

5.2.2 $1,25(OH)_2D3$ is less inhibitory on Treg as compared to T effector cell proliferation

Foxp3 has been identified as an early activation marker, as low levels of expression of Foxp3 are seen post-stimulation in CD4+ T cells [97]. To establish the effects of $1,25(OH)_2D3$ on existing Foxp3+ Tregs versus effects on conversion of effector T cells to Foxp3+ cells, Tregs and T effector CD4+ T cells were isolated from the peripheral blood by a cell sorter method (Figure 5-4).

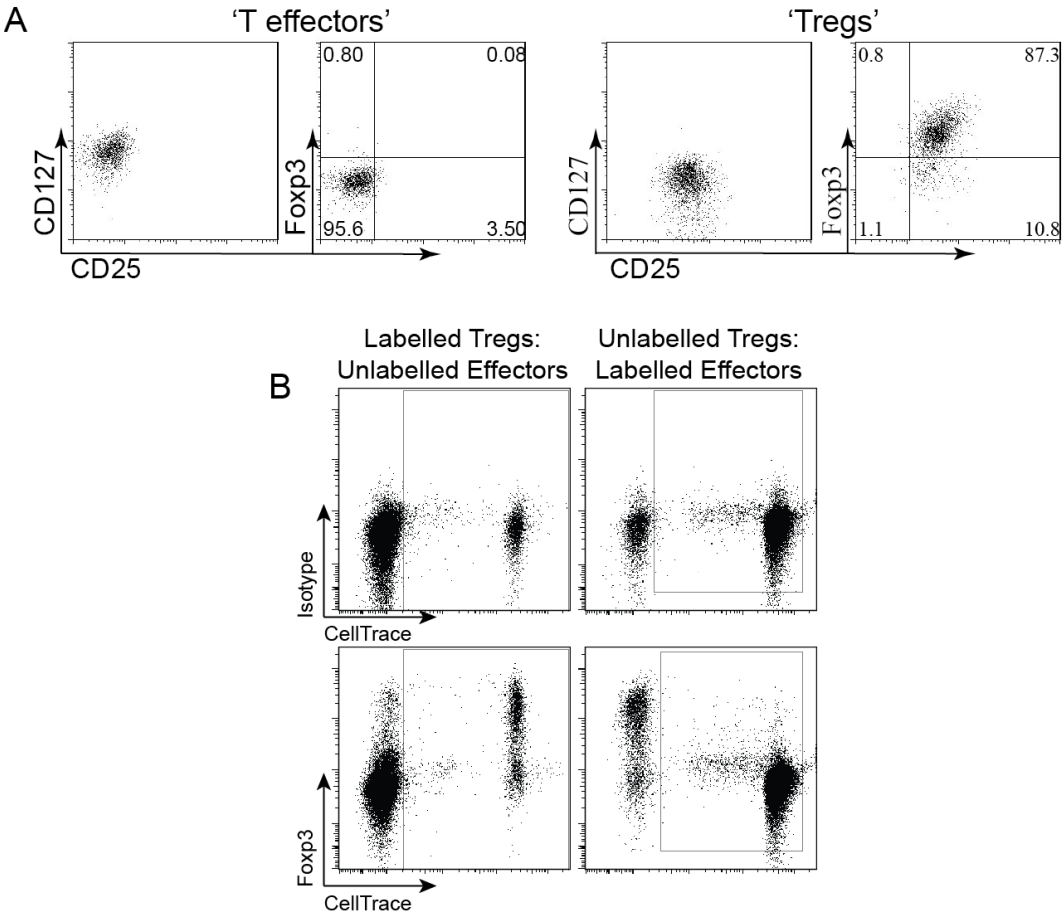


Figure 5-4 Sorting strategy to isolate Tregs and Teffectors

CD4+ cells were negatively selected from peripheral blood, and were subsequently cell surface stained with CD127 and CD25. **A,** Teffector cells (CD127hiCD25low) and Tregs (CD127loCD25hi) were FACs sorted based on their CD127 and CD25 expression, Teffector cells and Tregs were subsequently stained for Foxp3 (right). **B,** representative plots of cells cultured with anti-CD3 and IL-2 for 7-days in the presence of 10^{-6}M $1,25(OH)_2D3$, either the Tregs (right) or Teffectors (left) were labeled with CellTrace to assess proliferation in the respective populations of cells.

Tregs were sorted based on CD127loCD25hi expression, and Teffector cells were identified as being CD127hiCD25lo/-. Typically 87% of the Tregs isolated were Foxp3+, whereas <1% of the T effector cells were Foxp3+ (Figure 5-4A). To establish the effect of $1,25(OH)_2D3$ on Treg versus Teffector proliferation, either the Treg population or the

T effector population were labelled with CellTrace violet and then the two cell populations were reconstituted and cells were reconstituted at a 1 Treg to 9 T effector ratio (Figure 5-4B). Cells were cultured for 7-days with anti-CD3 and IL-2 in the presence or absence of $1,25(OH)_2D3$ at concentrations indicated, proliferation was assessed by loss of fluorescence of CellTrace. At Day 7 intranuclear Foxp3 staining was performed.

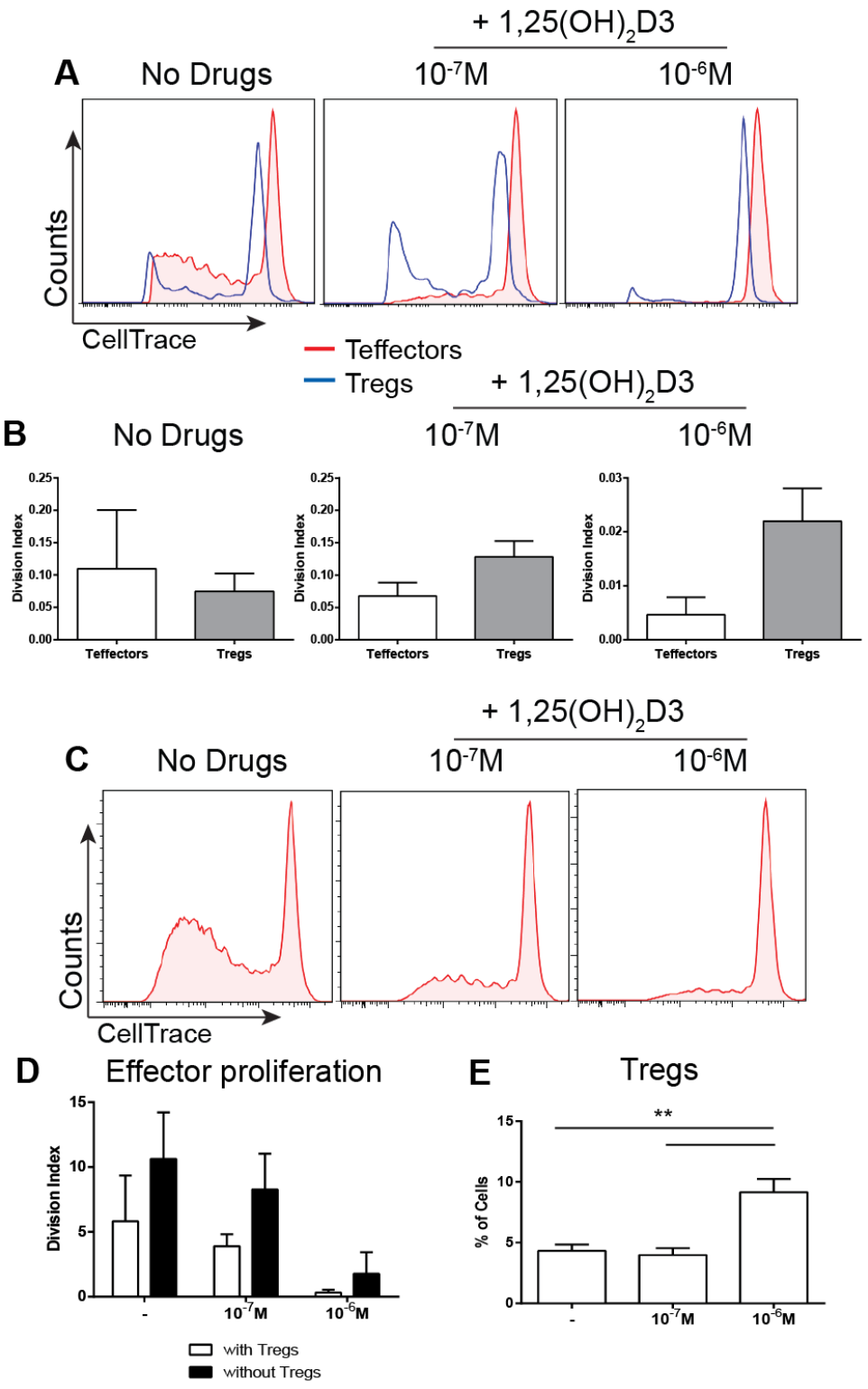


Figure 5-5 1,25(OH)$_2$D3 is less inhibitory on Treg as compared to T effector cell proliferation

CD4+ T Cells were isolated by negative selection then were further sorted based on CD25 and CD127 cell surface staining for Tregs (CD127loCD25hi) and T effectors (CD127hiCd25lo/-).

Either Tregs or Teffectors were labelled with CellTrace violet and were cultured together at the ratio 1:9 (Treg:Teffectors) and stimulated for 7-days with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of $1,25(OH)_2D3$ (VitD3; 10^{-X}M). Proliferation was assessed by CellTrace loss in fluorescence **A,** representative histograms of Teffector cells (CD127hiCD25low; red) versus Treg (CD127lowCD25+; blue) **B,** cumulative data of division index of Teffectors (white) versus Tregs(grey) (n=3) **C,** representative histograms of Teffector cells (CD127hiCD25low; red) when cultured alone **D,** cumulative data (n=4) with effectors cultured in the presence (white) or absence (black) of Tregs (n=4) **E,** frequency of Tregs post 7-days of culture cumulative data (n=4). **E,** differences in Treg frequencies under different culture conditions were assessed by repeated measures one-way ANOVA with Tukey's post-test. * $p \leq 0.05$, ** $p \leq 0.01$.

$1,25(OH)_2D3$ promoted Treg proliferation over T effector proliferation at both 10^{-7}M and 10^{-6}M (Figure 5-5A-B). However only three experiments were performed and although all demonstrated the same pattern significance was not achieved. Interestingly when effector cells were cultured in the absence of Tregs, $1,25(OH)_2D3$ seemed to be less inhibitory of effector proliferation (Figure 5-5C-D). Also of interest when the frequency of Tregs was assessed at Day 7, even though 10% Tregs were put in the system only around 5% were seen in the No drug and 10^{-7}M $1,25(OH)_2D3$ conditions. Whereas 10^{-6}M $1,25(OH)_2D3$ had significantly more Tregs as defined by CellTrace labelling as compared to the other conditions (Figure 5-5E). This could be due to the inhibitory effect of 10^{-6}M $1,25(OH)_2D3$ on T effectors, and/or due to additional mechanism(s) that prevents death and/or maintains Treg cells.

5.2.3 10^{-6}M $1,25(OH)_2D3$ increases the frequency of IL-2+ CD4+ T cells

IL-2 was originally described as a T cell growth factor. However IL-2 knockout mice have been shown to develop a lethal lymphoproliferative disease suggesting a lack of regulatory pathways in these mice [438,439]. Foxp3+ Tregs express high levels of the

CD25 receptor, the high-affinity form of the IL-2R [440-442]. High concentrations of IL-2 are require to expand Foxp3+ Tregs in culture [443]. However Foxp3+ Tregs are known to have an inability to make IL-2 themselves and hence rely on IL-2 production from other T effector cells [151]. As 10^{-6}M $1,25(OH)_2D3$ has been shown to be less inhibitory of Tregs as compared to T effectors, the question arose what role does IL-2 play in this effect. In initial experiments to address this IL-2 production was investigated by intracellular cytokine staining and CBA.

Cells were cultured for 3, 7 or 14 days with anti-CD3 and IL-2 in the presence or absence of drugs. At day 3, 7 or 14 PMA and Ionomycin was added for 4 hours, with the final 2-hours containing Monensin. Intracellular cytokine production was assessed by flow cytometry. Cumulative supernatants were collected at Day 7 and IL-2 production was assessed by Cytometric Bead Array (CBA).

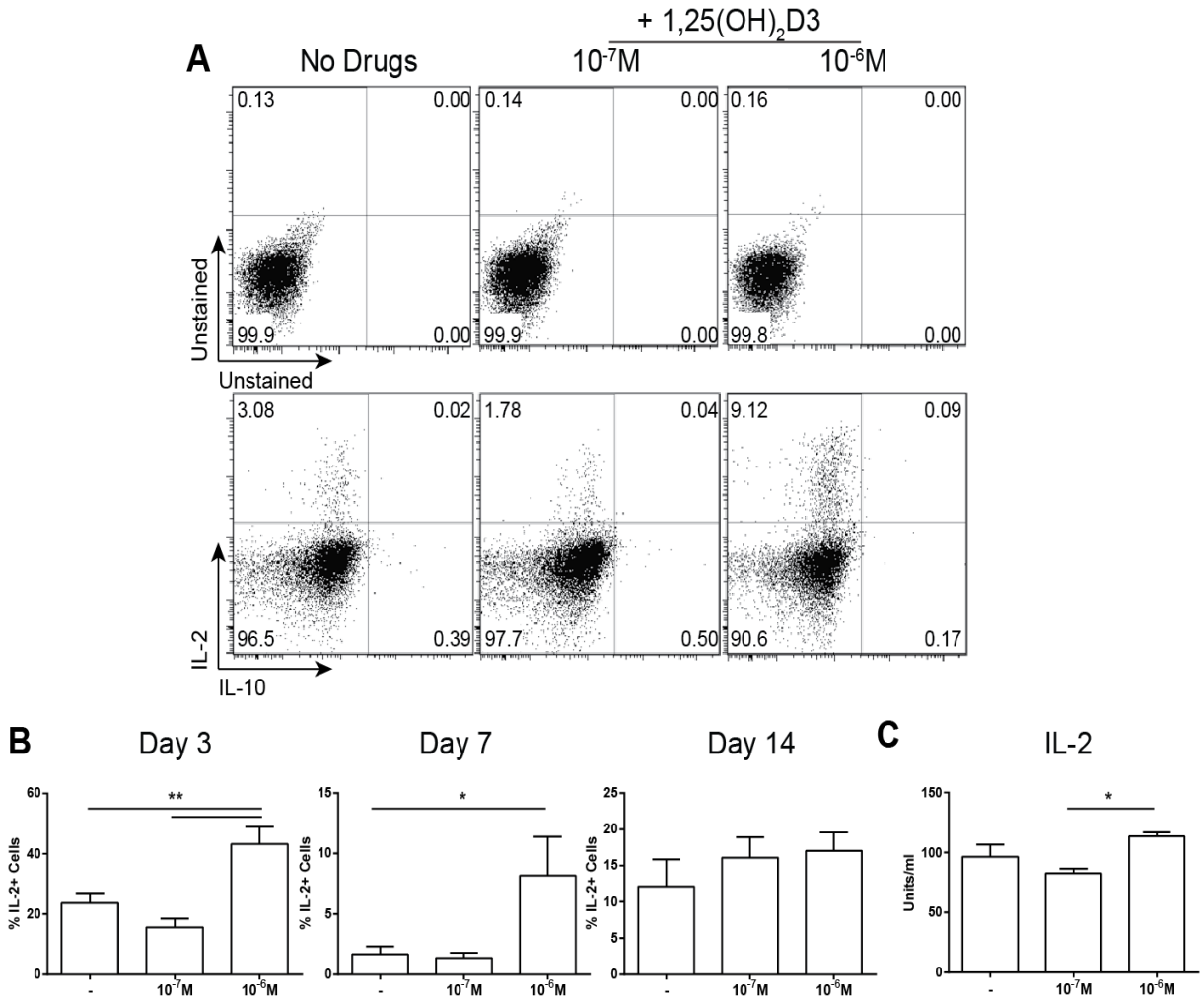


Figure 5-6 A high concentration of 1,25(OH)$_2$D3 (10^{-6}M) increases the frequency of IL-2+ CD4+ T cells

CD4+ T Cells stimulated for 3, 7 or 14 days with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-x}M). Intracellular cytokine staining was performed following 4 hours stimulation with PMA/Ionomycin with the final 2 hours containing Monensin. **A,** representative dot plots from Day 7 **B,** cumulative data of frequency of IL-2+ cells at day 3 (n=4), day 7 (n=5) and day 14 (n=3) **C,** Cumulative data of IL-2 as detected by CBA of supernatants collected post 7-days of culture. **B, and C,** differences in IL-2 production under different culture conditions assessed by a repeated measures one-way ANOVA with Tukey's post-test. * $p \leq 0.05$, **$p \leq 0.01$.

10^{-6}M 1,25$(OH)_2$D3 significantly increased the frequency of IL-2+ CD4+ T cells at Day 3 and Day 7 as compared to the no drug condition (Figure 5-6A-B). By Day 14 there was no significant difference in the frequency of IL-2+ CD4+ T cells between the conditions. Also there was an increased amount of IL-2 in the Day 7 supernatants as assessed by CBA in cultured that contained 10^{-6}M 1,25$(OH)_2$D3 (Figure 5-6C). Since exogenous IL-2 was added to the cultures the CBA data are difficult to interpret, however all values are above the 50IU added at the start of culture (Figure 5-6C).

5.2.4 IL-2 signalling predominantly occurs in Foxp3+ Tregs over Foxp3- T effector cells in cultures containing 10^{-6}M 1,25$(OH)_2$D3

The increase in IL-2 seen at 10^{-6}M 1,25$(OH)_2$D3 corresponds with the increased frequency of Foxp3+ Tregs (Figure 5-1 and 5-6) suggesting that IL-2 is a requirement of Foxp3+ Tregs. To further understand which cells were utilising the IL-2 in the 10^{-6}M 1,25$(OH)_2$D3 cultures, cell surface staining for CD25 (the high affinity receptor for IL-2) was performed. Total CD4+ cells (Figure 5-7A), or co-culture of Tregs plus Teffectors [at 1:9 ratio respectively] (Figure 5-7B) were cultured for 7 days with anti-CD3 and IL-2 in the presence or absence of drugs. At Day 7 cell surface staining for CD25 was performed with intranuclear Foxp3 staining.

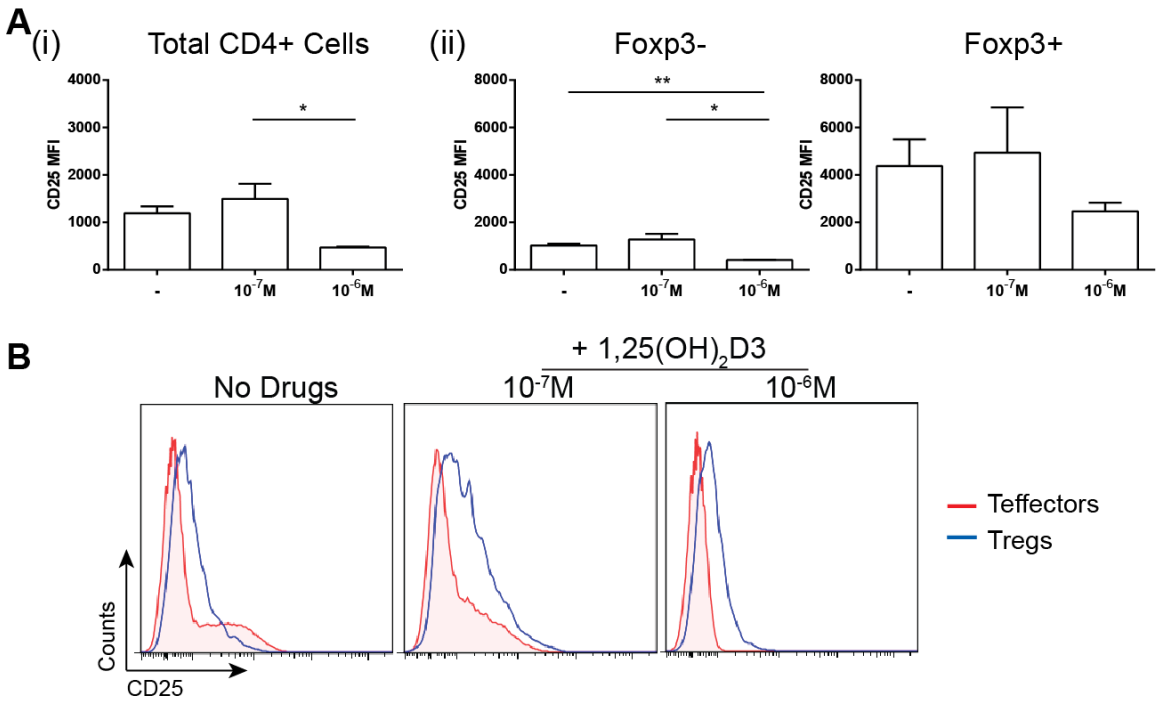


Figure 5-7 CD25 expression is significantly inhibited by 1,25(OH)$_2$D3 at 10^{-6}M on Foxp3- CD4+ T cells but not Foxp3+ CD4+ T cells

Figure 5-7A total CD4+ T Cells stimulated for one 7-day cycle; **Figure 5-7B** either Tregs or Teffectors were labelled with CellTrace violet. Cells were cultured at the ratio 1:9 (Treg:Teffectors) with either Treg or Teffector cells labeled with CellTrace violet as described in Figure 5-4. Cells were cultured for 7-days with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-x}M). **A,** Cumulative data of CD25 expression on (i) Total CD4+ Cells or (ii) Foxp3+ or Foxp3- cells CD4+ T cells **B,** representative histograms (of n=2) of T effectors (red) or Teffectors (blue) cultured as described in Figure 5-4 **A,** differences in CD25 expression under different culture conditions were assessed by a repeated measures one-way ANOVA with Tukey's post-test. * $p \leq 0.05$, ** $p \leq 0.01$.

There were significantly lower levels of CD25 on CD4+ T cells cultured in the presence of 10^{-6}M 1,25(OH)$_2$D3 as compared to 10^{-7}M 1,25(OH)$_2$D3 (Figure 5-7Ai). When the cells were assessed according to their Foxp3 expression, there was significantly lower expression of CD25 in the Foxp3- CD4+ T cell population in the presence of 10^{-6}M 1,25(OH)$_2$D3 as compared to 10^{-7}M 1,25(OH)$_2$D3 and no drug condition. In contrast

there was no significant difference in CD25 expression on Foxp3+ CD4+ T cells under all experimental conditions (Figure 5-7Aii). To confirm that the Foxp3+ cells seen in Figure5-7A were Tregs, CD25 expression was assessed on Tregs and T effectors individually labelled (isolated and cultured as described in Figure 5-4). 10^{-6}M $1,25(OH)_2D3$ seemed to prevent the upregulation of CD25 on T effector cells in culture as compared to no drug and 10^{-7}M $1,25(OH)_2D3$ conditions, whereas the expression of CD25 on Tregs seemed to be similar in all culture conditions (Figure 5-7B).

To confirm that the IL-2 was being preferentially used by the Tregs in cultures containing 10^{-6}M $1,25(OH)_2D3$ and not the T effectors cells, phosphorylation of STAT5 as a marker of IL-2 signalling was assessed. Downstream of IL-2 ligation of CD25 there is phosphorylation of tyrosine at site 694, which is a prerequisite for STAT5 activation [444-446]. Treg or T effector cells were labelled with CellTrace (Figure 5-4B) and were cultured at a 1 Treg to 9 Teffector ratio. Cells were cultured for 7-days with anti-CD3 and IL-2 in the presence or absence of $1,25(OH)_2D3$ at concentrations indicated. At Day 7 intracellular staining for phospo-STAT5 was performed.

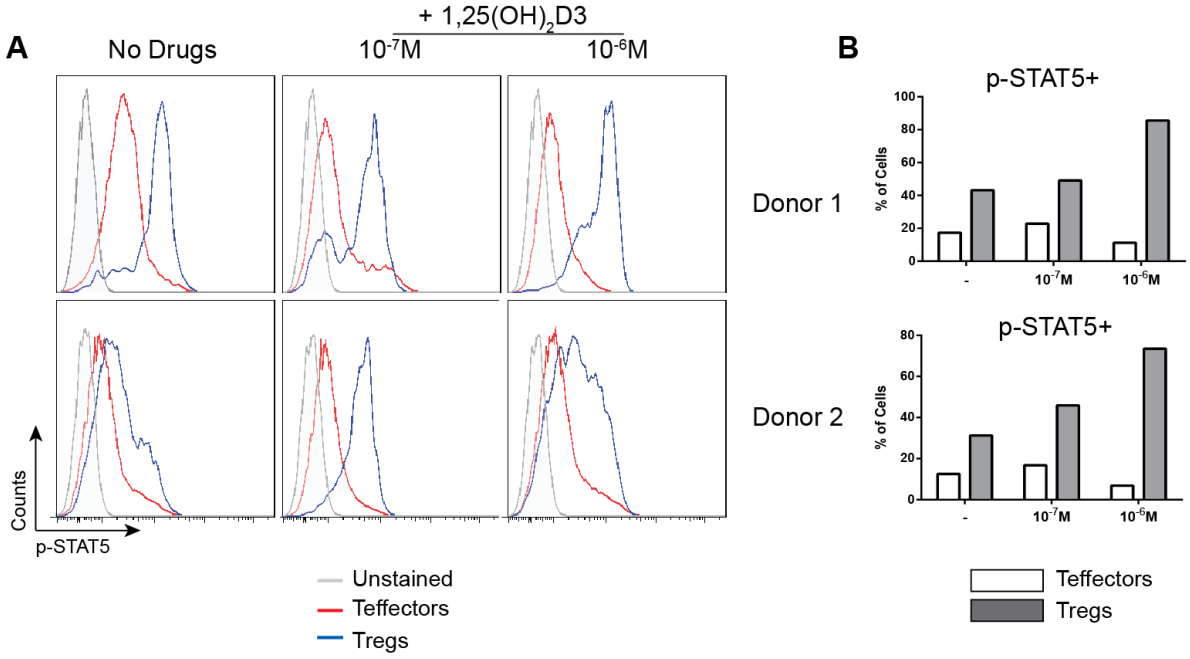


Figure 5-8 IL-2 signaling predominantly occurs in Tregs in cultures containing 10^{-6}M $1,25(OH)_2D3$

CD4+ T Cells were isolated by negative selection then were further sorted based on CD25 and CD127 cell surface staining for Tregs (CD127loCD25hi) and T effectors (CD127hiCD25low) (as described in Figure 5-4). Either Tregs or Teffectors were labelled with CellTrace violet and were cultured at the ratio 1:9 (Treg:Teffectors) and stimulated for 7-days with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of $1,25(OH)_2D3$ (VitD3; 10^{-X}M). A, histograms of two donors showing phospo-STAT5 (p-STAT5) staining in Teffectors (red) and Tregs (blue) and unstained (grey) B, frequency of phospo-STAT5+ (p-STAT5+) cells (gating based on unstained cells) Teffectors (white) and Tregs (grey) post 7-days of culture.

In all conditions there was more IL-2 signalling, as determined by phosphorylation of STAT5, in the Tregs as compared to Teffectors. However in cultures containing 10^{-6}M $1,25(OH)_2D3$ there was much more phosphorylation of STAT5 in Tregs and less in Teffectors as compared to the no drug and 10^{-7}M $1,25(OH)_2D3$ conditions (Figure 5-8). This data suggests that the lack of upregulation of CD25 seen in Figure 5-7 in cultures

containing 10^{-6}M 1,25$(OH)_2$D3 results in the IL-2 being preferentially consumed by the Tregs.

5.2.5 1,25$(OH)_2$D3 increases TGFβ expression in CD4+ T cell cultures

TGFβ has been shown to be an important factor for induction of Foxp3+Tregs, in particular for iTregs [178]. Earlier data in the lab showed that the addition of anti-TGFβ in 10^{-6}M 1,25$(OH)_2$D3 containing cultures reduced the frequency of Foxp3+ CD4+ T cells [342]. The next aim was to further investigate the role of TGFβ in 1,25$(OH)_2$D3-induced Treg induction, and the capacity of vitamin D to modulate TGFβ levels.

Attempts were made to detect TGFβ protein by ELISA and CBA but we were not satisfied with the data generated from the assays (data not shown). So two surrogate markers of TGFβ expression were used Latency Associated Protein (LAP) and Glycoprotein A repetitions predominate (GARP) to assess protein production of TGFβ [447]. TGFβ is synthesised as a propeptide, it is further activated through cleavage of the N-terminal pro-peptide from the TGFβ by the protein FURIN, and this N-terminal propeptide is LAP [448-450]. Subsequently, LAP non-covalently binds to the active TGFβ and it is co-expressed on the surface of cells. LAP prevents the binding of TGFβ to its target receptor [450]. GARP is a transmembrane protein expressed on the surface of cells and is a receptor for LAP bound to TGFβ. GARP has been proposed to play a role in anchoring LAP-TGFβ onto the cell surface as well as activating TGFβ itself [447,451,452]. Both LAP and GARP have been shown to be specifically expressed on Foxp3+ Tregs [451-453].

TGFβ gene expression as well as cell surface LAP and GARP expression was assessed in CD4+ T cell cultures. CD4+ cells were cultured for either one or two 7-day rounds of culture with anti-CD3 and IL-2 in the presence or absence of 1,25$(OH)_2$D3. At Day 7 and Day 14 cell pellets were obtained and gene expression of TGFβ was

assessed by qRT-PCR. Also at Day 7 and Day 14 cell surface expression of LAP and GARP was assessed.

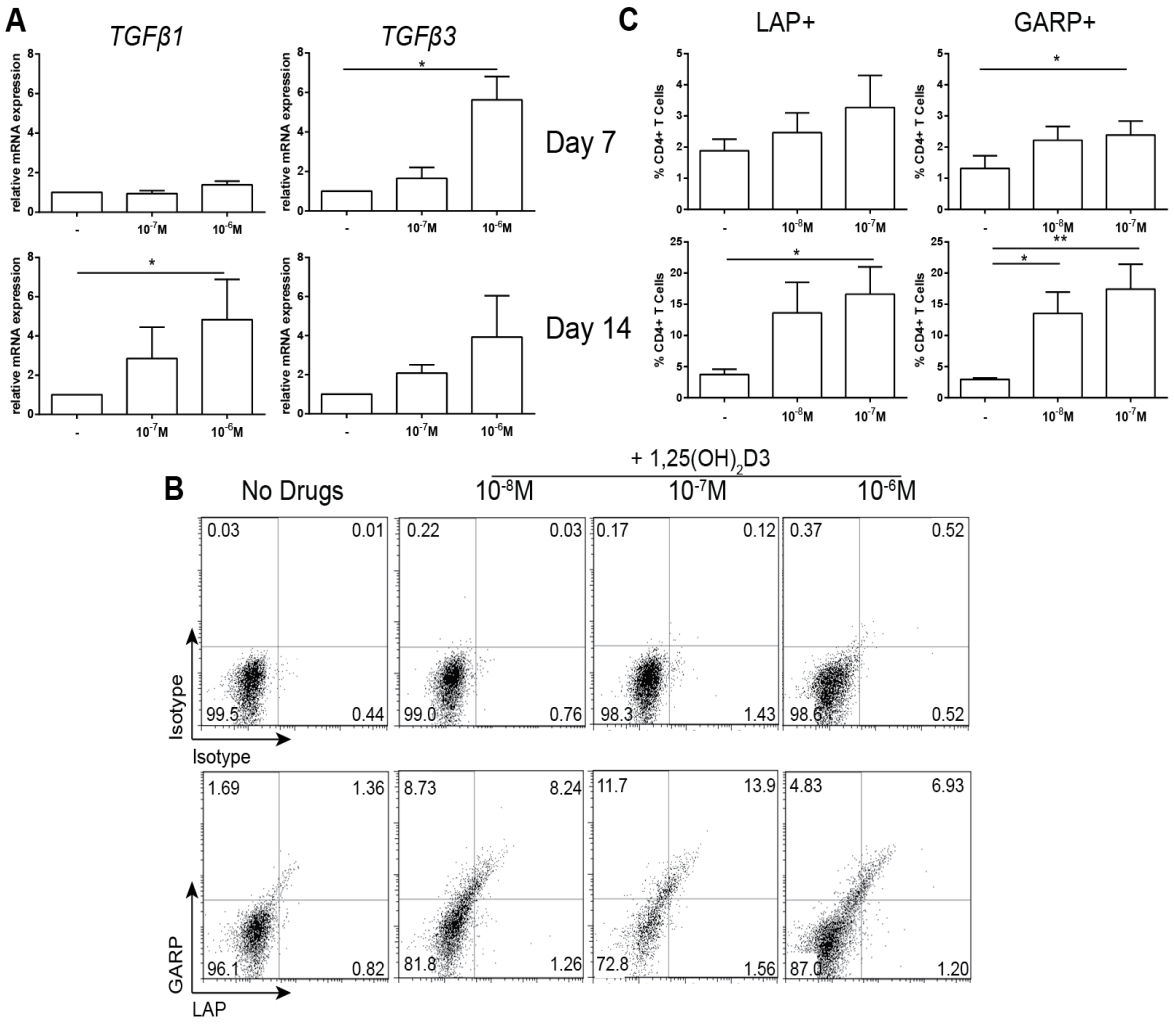


Figure 5-9 1,25(OH)$_2$D3 upregulates TGFβ gene and protein expression

CD4+ T Cells stimulated for one or two 7-day cycles with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-X}M). **A,** cumulative data of relative mRNA expression of TGFβ1 and TGFβ1 gene expression Day 7 (top; n=5) and Day 14 (bottom; n=4) **B,** representative dot plots of CD4+ cells cell surface stained for LAP and GARP at Day 14 **C,** Cumulative data of GARP and LAP expression at Day 7 (top; n=5) and Day 14 (bottom; n=4). **B,** differences in mRNA gene expression under different culture conditions were assessed by Freidman test with Dunn's multiple comparison post-test **C,** differences in LAP and GARP expression under different culture conditions was assessed by a repeated measures one-way ANOVA with Tukey's post-test. * $p \leq 0.05$, ** $p \leq 0.01$.

There are three isoforms of TGFβ; TGFβ1, TGFβ2, and TGFβ3 with TGFβ1 being the most common and dominant isoform in the immune system [454,455]. The majority of research on the role of TGFβ in the immune system has focussed on the predominant isoform TGFβ1 [159,178]. *TGFβ2* was not detected in CD4+ T cells at any time-points sampled (data not shown). 1,25$(OH)_2$D3 at 10^{-6}M significantly increased the expression of *TGFβ3* at Day 7 and a similar trend was seen at Day 14. Conversely, *TGFβ1* was not significantly altered by 1,25$(OH)_2$D3 at day 7, whereas by Day 14 there was a significant increase in *TGFβ1* gene expression as compared to the no drug condition (Figure 5-9A). Cell surface expression of LAP and GARP were used as surrogate markers for TGFβ expression. 1,25$(OH)_2$D3 significantly increased the expression of LAP and GARP on CD4+ T cells after 14 days of culture (Figure 5-9B and C). The optimum concentration for increasing LAP and GARP expression was 10^{-7}M 1,25$(OH)_2$D3, and there was a significant increase in GARP expression at Day 7 as well as Day 14 along with LAP (Figure 5-9C).

To further characterise these LAP and GARP populations Foxp3 intranuclear staining was also performed. At Day 7 and Day 14 cell surface expression of LAP and GARP was assessed with intranuclear Foxp3 staining.

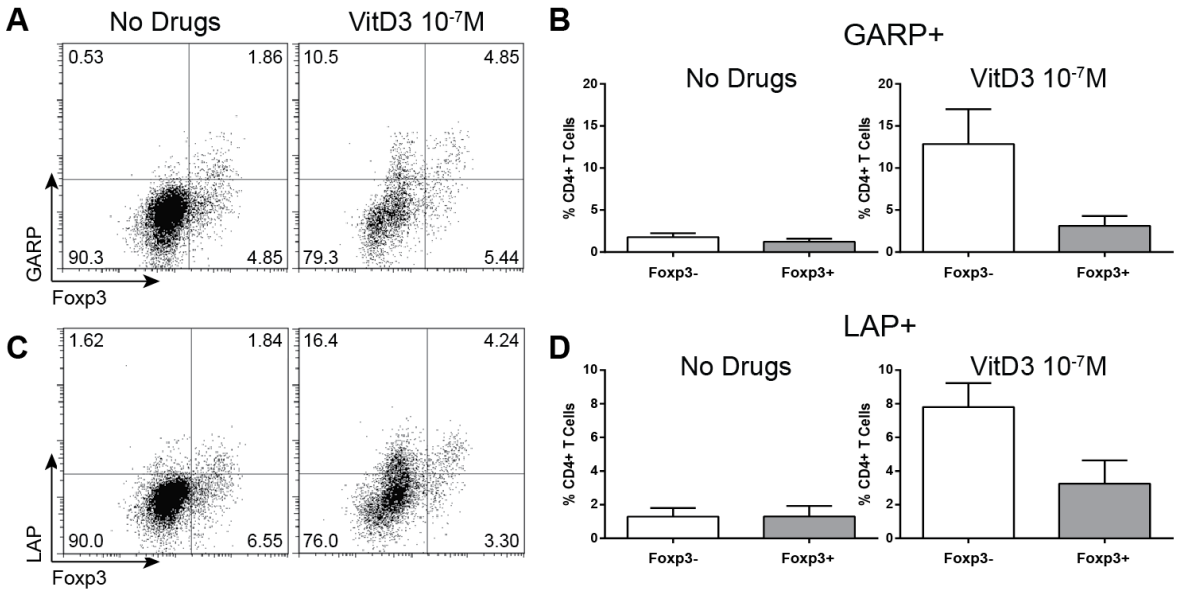


Figure 5-10 1,25(OH)$_2$D3 predominantly upregulates LAP and GARP in Foxp3- CD4+ T cells

CD4+ T Cells stimulated for two 7-day cycles with anti-CD3 and IL-2 (No drugs) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-x}M). **A,** representative dot plots and **B,** Cumulative data of GARP expression in Foxp3- (white) and Foxp3+ (grey) populations (n=3) **C,** representative dot plots and **D,** Cumulative data of LAP expression in Foxp3- (white) and Foxp3+ (grey) populations (n=3).

In the no drug condition there is no significant difference in GARP and LAP expression between Foxp3+ CD4+ T cells and Foxp3- CD4+ T cells. The addition of 10^{-7}M 1,25(OH)$_2$D3 into culture resulted in a trend towards an increase in expression of LAP and GARP ($p = 0.079$ and $p = 0.133$ respectively) on the surface of Foxp3- CD4+ T cells as compared to Foxp3+ CD4+ T cells. The addition of 1,25(OH)$_2$D3 resulted in increased expression of LAP and GARP on Foxp3+ and Foxp3- cells, the majority of the upregulation is occurring in the Foxp3- CD4+ population (Figure 5-10).

5.2.6 TGFβ increases the frequency of Foxp3+ CD4+ T cells in culture in the presence of a lower concentration of $1,25(OH)_2D3$

Although 10^{-6}M $1,25(OH)_2D3$ may be applicable in some environments as physiological, emerging data suggests that 10^{-7}M $1,25(OH)_2D3$ may be more physiological [272,273]. It was shown that human monocyte-derived Dendritic cells had the capacity to generate $1,25(OH)_2D3$ from 25(OH)D at concentrations ranging from 10^{-9}M to 10^{-8}M in culture [272,273]. As the optimum induction of LAP and GARP occurs at 10^{-7}M $1,25(OH)_2D$, thus the question remains - what is the function of TGFβ, and does it play a role in Treg induction? To answer this, TGFβ was added to culture with the lower concentration of $1,25(OH)_2D3$ to establish the effects of TGFβ on Foxp3+ Treg frequencies.

CD4+ cells were stimulated with anti-CD3 and IL-2 in the presence or absence of drugs ($1,25(OH)_2D$ 10^{-7}M; TGFβ 2ng/ml). At Day 7 and Day 14 cells were stained for intranuclear Foxp3 expression.

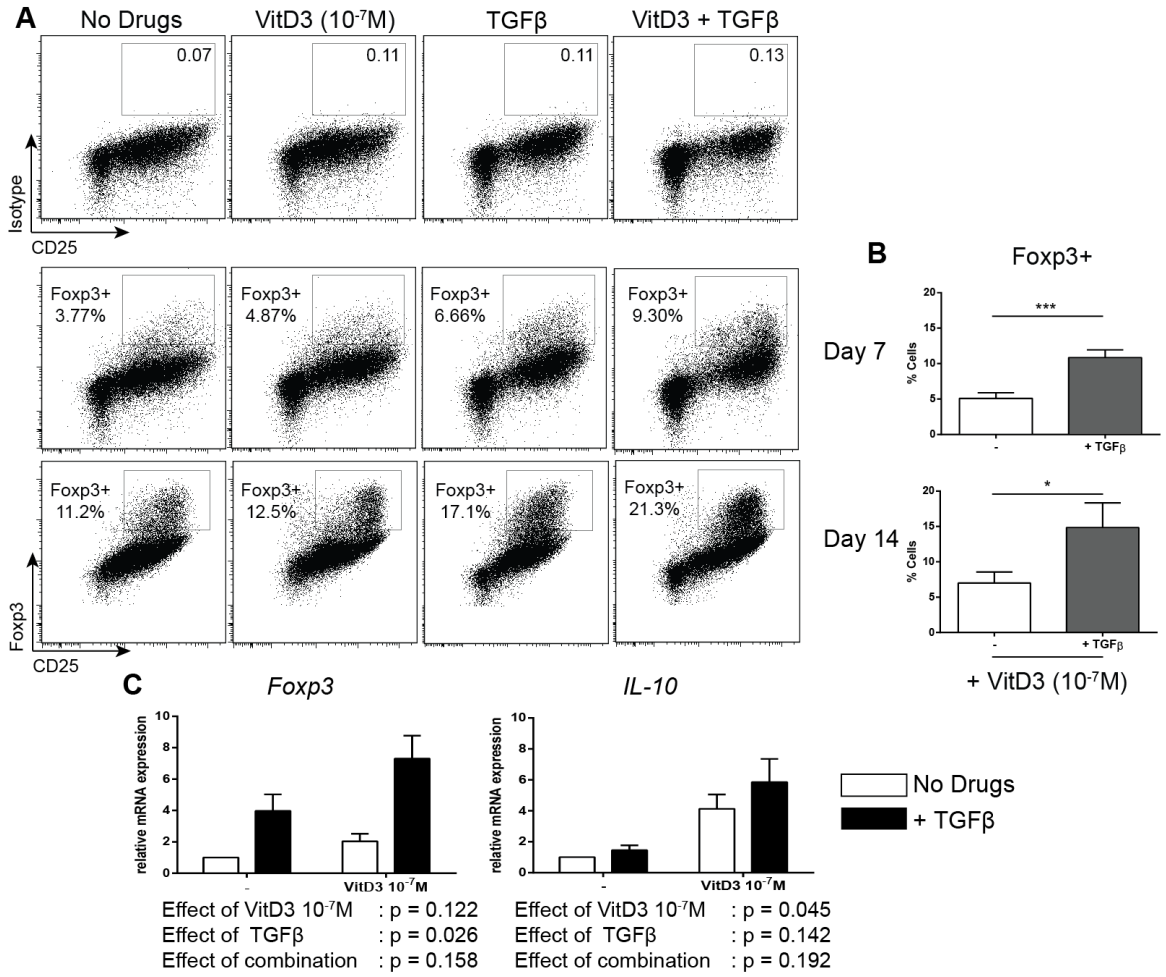


Figure 5-11 1,25(OH)$_2$D3 and TGFβ increase the frequency of Foxp3+ CD4+ T cells

CD4+ T Cells stimulated for two 7-day cycles with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-X}M) and/or TGFβ (2ng/ml) . **A,** representative dot plots of Foxp3 expression in CD4+ T cells cultured in the presence or absence of 1,25(OH)$_2$D3 and/or TGFβ as compared to isotype control **B,** Cumulative data of CD4+ T cells cultured in the presence of 1,25(OH)$_2$D3 (VitD3 10-7M) in the absence (white) or presence (grey) of TGFβ (n=4). **C,** cumulative data of relative mRNA expression of Foxp3 and IL-10 gene expression at Day 7 (n=4). **B,** differences in the frequency of Foxp3+ CD4+ T cells under different culture conditions was assessed by paired t-test and **C,** differences in mRNA expression under different culture conditions was assessed by a repeated measures two-way ANOVA with Sidak's multiple comparison test.* $p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

As previously reported 10^{-7}M 1,25$(OH)_2$D does not significantly increase the frequency of Foxp3+ CD4+ T cells at either Day 7 or Day 14 as compared to no drug. When TGFβ is present in culture there is a significant increase in the frequency of Foxp3+ T cells, as has been reported previously. The addition of TGFβ to 10^{-7}M 1,25$(OH)_2$D containing cultures significantly increased the frequency of Foxp3+ T cells at Day 7 and Day 14 (Figure 5-11A and B). When the gene expression was assessed TGFβ had a significant effect of the expression on Foxp3, there was no additional synergistic effect of the combination of TGFβ and 10^{-7}M 1,25$(OH)_2$D (Figure 5-11C). As expected 10^{-7}M 1,25$(OH)_2$D but not TGFβ significantly increased the gene expression of IL-10 from CD4+ T cells.

To try and establish the mechanism behind the increased frequency of Foxp3+ T cells in culture in the presence of 10^{-7}M 1,25$(OH)_2$D and TGFβ, proliferation was assessed in Foxp3+ and Foxp3- populations. CD4+ T cells were labeled at Day 0 with CellTrace and cultured for one or two 7-day rounds of stimulation with anti-CD3 and IL-2 in the presence or absence of drugs (1,25$(OH)_2$D 10^{-7}M) and TGFβ (2ng/ml). Proliferation was assessed by loss of fluorescence of CellTrace. At Day 14 intranuclear Foxp3 staining was performed and samples were assessed by flow cytometry.

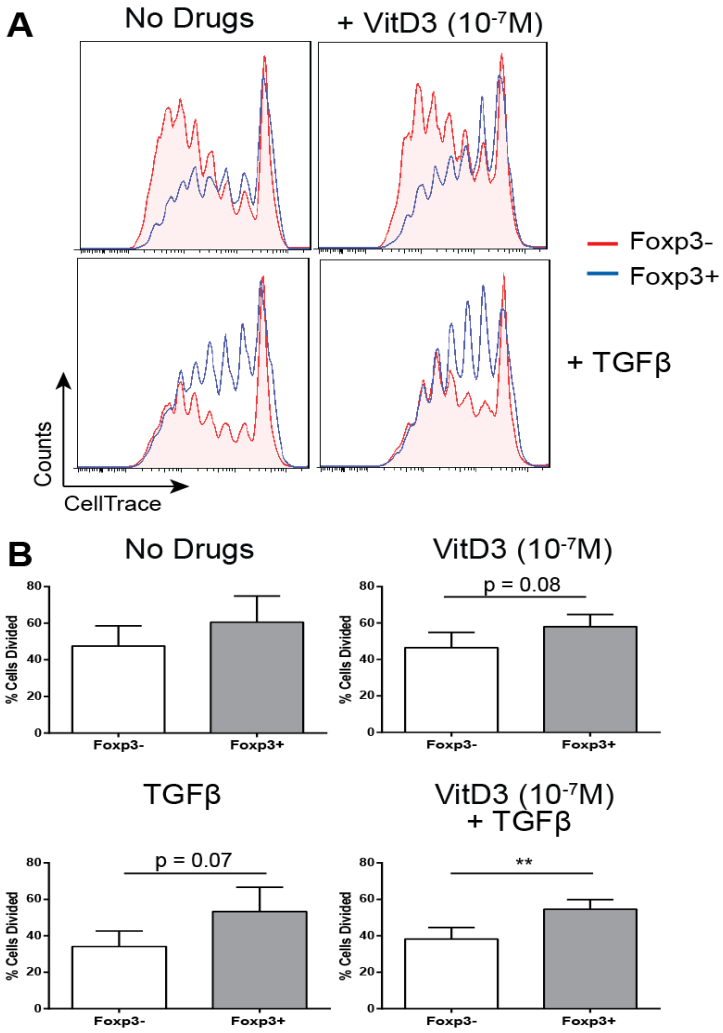


Figure 5-12 A combination of TGFβ and 10^{-7}M 1,25(OH)$_2$D3 is less inhibitory on proliferation of Foxp3+ as compared to Foxp3- CD4+ T cells

CD4+ T Cells stimulated for two 7-day cycles with anti-CD3 and IL-2 (No drugs; -) or additionally with the indicated concentration of 1,25(OH)2D3 (VitD3; 10^{-X}M) and/or TGFβ (2ng/ml) . Cells were labeled at Day 0 with CellTrace Violet and proliferation was assessed by loss in fluorescence. **A,** representative histograms of CellTrace expression in Foxp3- (red) and Foxp3+ (blue). **B,** Cumulative data of the percent of original population divided in Foxp3- (white) and Foxp3+ (n=4). **B,** differences in Foxp3+ and Foxp3- proliferation under different culture conditions were assessed by paired t-test * $p \leq 0.05$, ** $p \leq 0.01$.

Although the effect is modest there is significantly more proliferation in the Foxp3+ population as compared to the Foxp3- in cultures containing a combination of 10^{-7}M

1,25$(OH)_2$D and TGFβ. There was a similar trend seen in cultures that contained only TGFβ or 10^{-7}M 1,25$(OH)_2$D (Figure 5-12).

5.2.7 A combination of 10^{-7}M 1,25$(OH)_2$D3 and TGFβ greatly increases the frequency of IL-2+ CD4+ T cells in culture.

IL-2 was shown to be increased at the higher doses of 1,25$(OH)_2$D which coincided with an increased frequency of Foxp3+ T cells (Figure 5-6). IL-2 secretion was assessed in cultures containing TGFβ and 10^{-7}M 1,25$(OH)_2$D to investigate whether IL-2 production also coincides with increased Foxp3 expression in cultures containing TGFβ. CD4+ T cells were cultured for 3, 7 days with anti-CD3 and IL-2 in the presence or absence of 1,25$(OH)_2$D at 10^{-7}M and TGFβ at 2ng/ml). At day 3 and 7 PMA and Ionomycin was added for the final 4 hours of culture, with the last 2-hours containing Monensin. Intracellular cytokine production was assessed by flow cytometry.

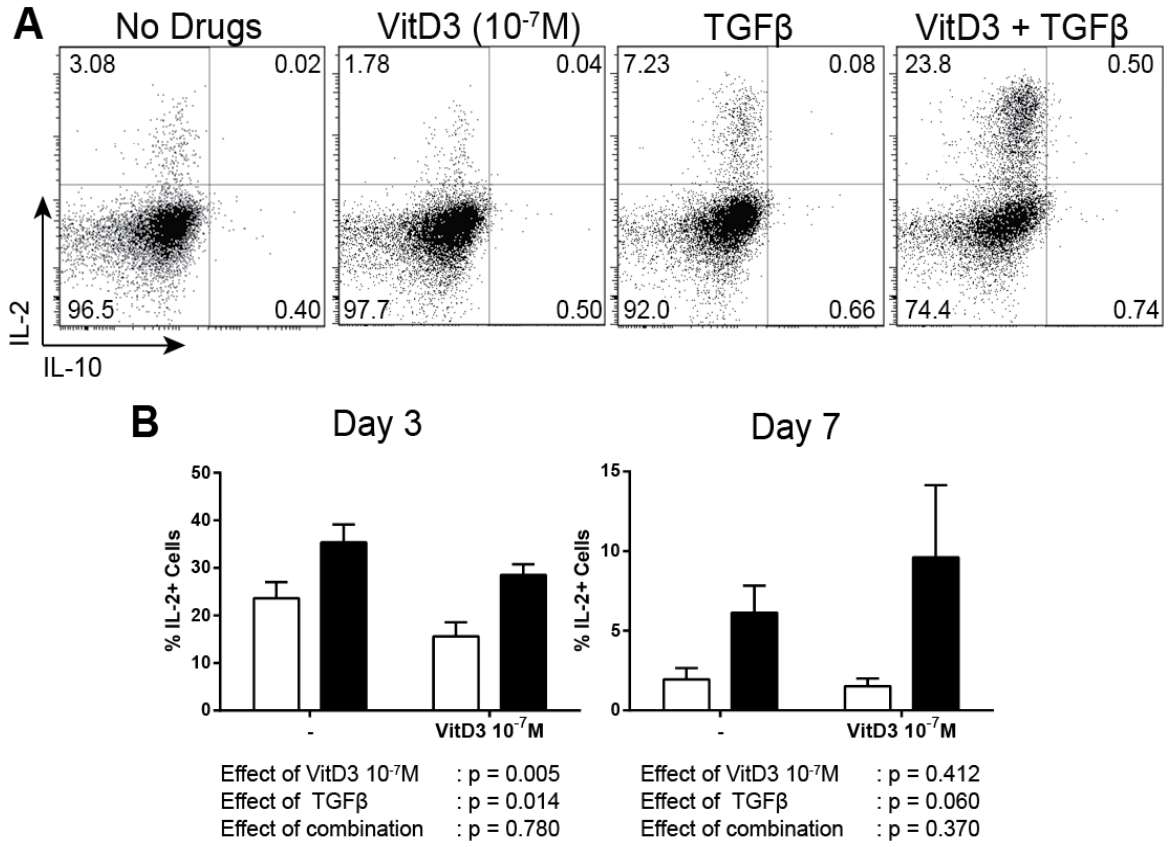


Figure 5-13 A combination of TGFβ and 10^{-7}M $1,25(OH)_2D3$ increase the frequency of IL-2+ CD4+ T cells

CD4+ T Cells stimulated with anti-CD3 and IL-2 (50 IU/ml) for 3 or 7 days (No drugs) or additionally with the indicated concentration of $1,25(OH)_2D3$ (VitD3; 10^{-x}M) and/or TGFβ (2ng/ml). Cells were stimulated for 4 hours with PMA/Ion with the final 2 hours containing monensin. **A,** representative dot plots of intracellular cytokine staining for IL-10 and IL-2 at Day 7 **B,** Cumulative data of IL-2+ cells in the absence (white) or presence (black) of TGFβ (n=4). **B,** differences in mRNA expression under different culture conditions was assessed by a repeated measures two-way ANOVA with Sidak's multiple comparison test.

10^{-7}M $1,25(OH)_2D$ alone significantly inhibited IL-2 at Day 3 but not Day 7 as compared to the no drug condition. Whereas TGFβ significantly increased IL-2 at Day 3 with a similar trend seen at Day 7 as compared to the no drug condition. There was no

synergy seen with the combination of 10^{-7}M 1,25$(OH)_2$D and TGFβ on IL-2+ cells, although considerably variability between donors was seen (Figure 5-13).

5.2.8 IL-10 inhibits the increase in frequency of Foxp3+ CD4+ Cells in cultures containing 1,25$(OH)_2$D

It has previously been shown that the addition of anti-IL-10R in cultures containing 10^{-7}M 1,25$(OH)_2$D increases the frequency of Foxp3+ CD4+ T cells in culture [342]. To assess whether the reverse could be true, IL-10 was added to cultures containing 10^{-6}M 1,25$(OH)_2$D and Foxp3+ CD4+ T cells were assessed. CD4+ cells were cultured for either one or two 7-day rounds of culture with anti-CD3 and IL-2 in the presence or absence of drugs (1,25$(OH)_2$D 10^{-6}M; IL-10 5ng/ml). At Day 7 gene expression was assessed by qRT-PCR and at Day 14 cells were stained for intranuclear Foxp3 expression and assessed by flow cytometry.

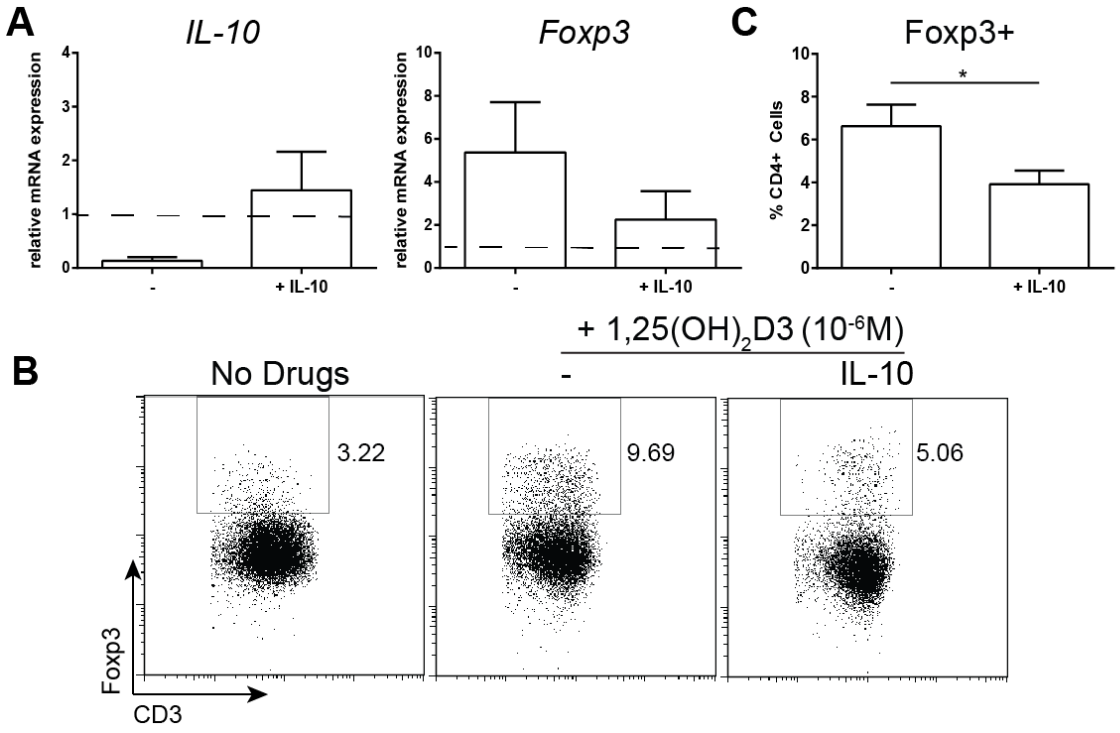


Figure 5-14 The addition of IL-10 in culture reduced the frequency of 1,25(OH)$_2$D3 (10^{-6}M)–induced Foxp3+ Tregs

CD4+ T Cells stimulated with anti-CD3 and IL-2 (50 IU/ml) for two 7-day cycles (No drugs; -) or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-x}M) and/or IL-10 (5ng/ml). **A,** cumulative data of relative mRNA expression of IL-10 and Foxp3 at day 7 (n=4) **B,** representative dot plots and **C,** cumulative data (n=5) of the frequency Foxp3+ CD4+ T cells at Day 14. **C,** differences in the % of Foxp3+ CD4+ T cells under different culture conditions was assessed by a paired t-test. * $p \leq 0.05$, ** $p \leq 0.01$. Dotted line indicated the levels of gene expression seen in CD4+ T cell cultures with no drugs present.

The addition of IL-10 in cultures containing 10-6M 1,25(OH)2D increased IL-10 and decreased Foxp3 gene expression back to similar levels to those of CD4+ T cells cultured in the presence of no drug (Figure 5-14A). There were significantly less Foxp3+ T cells in 10-6M 1,25(OH)2D cultures that contained IL-10 (Figure 5-14B and C).

Blockade of IL-10 signalling with anti-IL-10R blocking antibody has been shown previously in our lab to increase the frequency of Foxp3+ T cells [342], and effects on proliferation of Foxp3+ and Foxp3- CD4+ T cells was assessed. CD4+ T cells were

labeled at Day 0 with CellTrace and cultured for 7-days with anti-CD3 and IL-2 in the presence or absence of 1,25(OH)$_2$D at 10^{-7}M, in the presence or absence of anti-IL-10R or control antibody both at 5μg/ml. Proliferation was assessed by loss of fluorescence of CellTrace. At Day 7 intranuclear Foxp3 staining was performed and samples were assessed by flow cytometry.

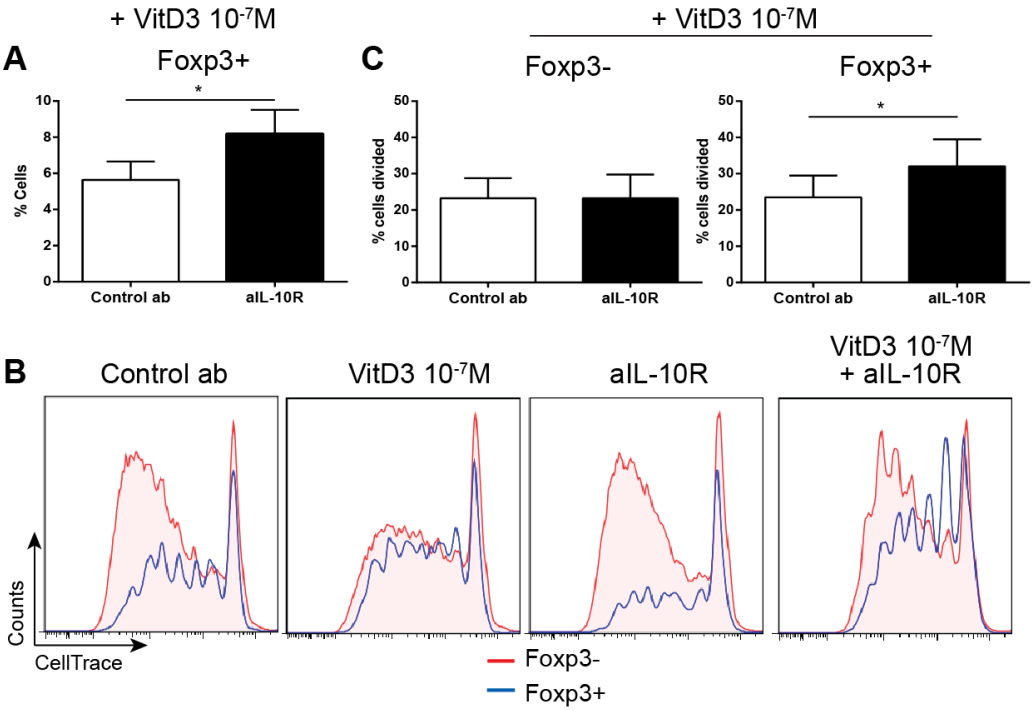


Figure 5-15 Anti IL-10R antibody increases the frequency of 1,25(OH)$_2$D3 (10^{-7}M)-induced Foxp3+ T cells by promoting the proliferation of Foxp3+ over Foxp3- CD4+T cells

CD4+ T Cells stimulated with anti-CD3 and IL-2 (50 IU/ml) for 7-days or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-x}M) and/or aIL-10-R antibody (5μg/ml) and/or IgG control antibody (5μg/ml). Cells were labeled at Day 0 with CellTrace Violet and proliferation was assessed by loss in fluorescence. **A,** cumulative data of Foxp3+ cells at day 7 (n=5) **B,** representative histograms of CellTrace labelling in Foxp3- (red) and Foxp3+ (blue) populations **C,** cumulative data (n=5) cells cultured with 1,25(OH)$_2$D3 10^{-7}M in Foxp3- (left) and Foxp3+ (right) in the absence (white) or presence (black) of anti-IL-10R. **A,** differences in % Foxp3+ CD4+ T cells under different culture conditions was assessed by a paired t-test and **C,**

differences in Foxp3+ and Foxp3- proliferation under different culture conditions were assessed by a paired t-test. * $p \leq 0.05$, ** $p \leq 0.01$.

In confirmation of earlier findings, the addition of anti-IL-10R into cultures containing 10^{-7}M $1,25(OH)_2D3$ significantly increased the frequency of Foxp3+ CD4+ T cells at Day 7 (Figure 5-15A). Anti-IL-10R increased proliferation of Foxp3+ CD4+ T cells in 10^{-7}M $1,25(OH)_2D3$ containing cultures (Figure 5-15B and C).

5.2.9 The combination of anti-IL-10R antibody, TGFβ and 10^{-7}M $1,25(OH)_2D3$ increases the frequency of Foxp3+ CD4+ T cells

As the addition of either anti-IL-10R or TGFβ in the presence of 10^{-7}M $1,25(OH)_2D3$ modestly but significantly enhanced the frequency of Foxp3+ CD4+ T cells, a combination of all was investigated. CD4+ T cells were labeled at Day 0 with CellTrace and cultured for one or two rounds of 7-day culture with anti-CD3 and IL-2 in the presence or absence of $1,25(OH)_2D$ at 10^{-7}M; anti IL-10R 5μg/ml; control antibody 5μg/ml; TGFβ 2ng/ml. Proliferation was assessed by loss of fluorescence of CellTrace.

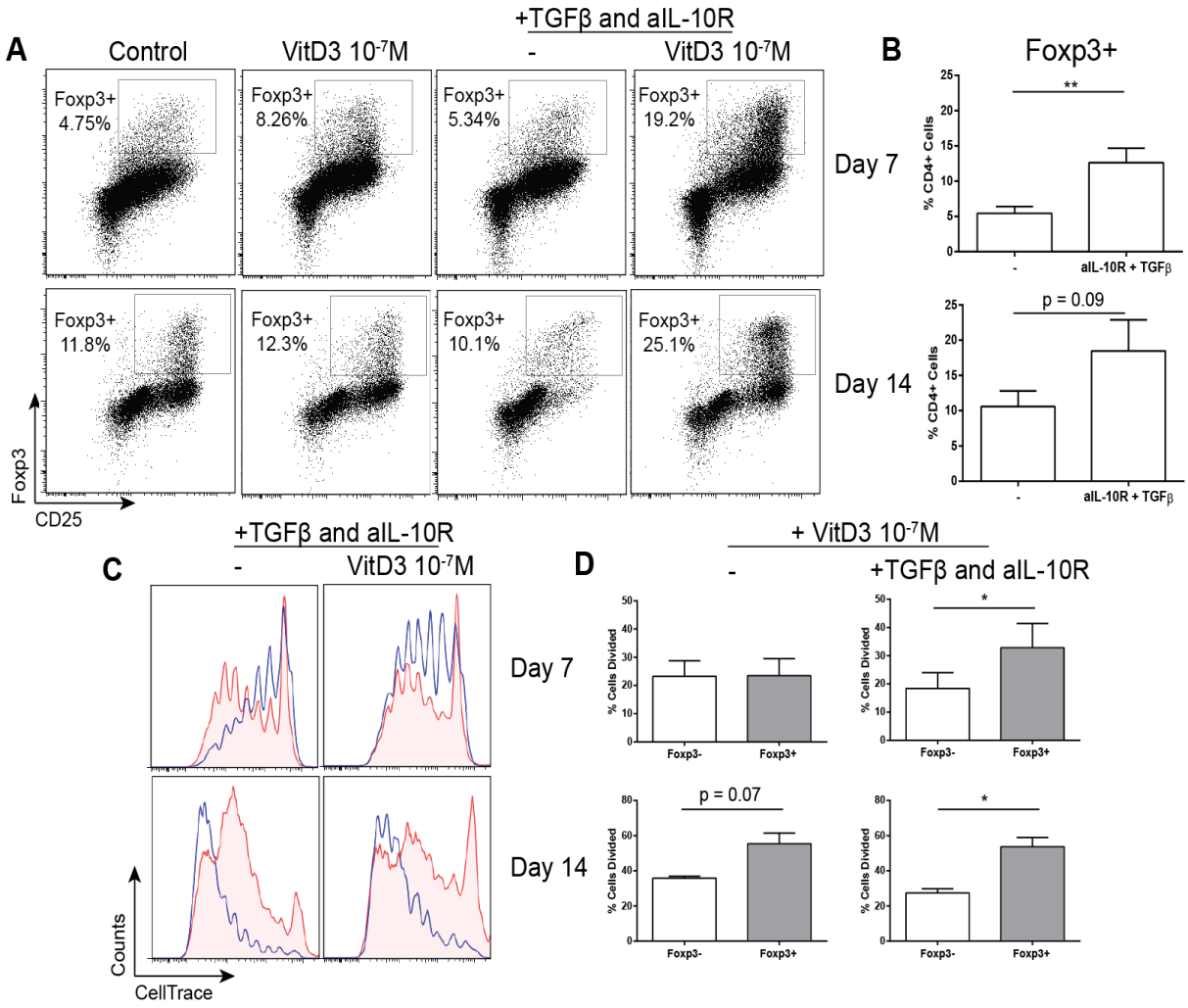


Figure 5-16 Combination of TGFβ and anti-IL-10R greatly increases the frequency of Foxp3+ Tregs at lower concentrations of 1,25(OH)$_2$D3

CD4+ T Cells stimulated with anti-CD3 and IL-2 for one or two 7-day cycles or additionally with the indicated concentration of 1,25(OH)$_2$D3 (VitD3; 10^{-x}M) and/or anti-IL-10-R antibody (5μg/ml) and/or IgG control antibody (5μg/ml) and/or TGFβ (5ng/ml). Cells were labeled at Day 0 with CellTrace Violet and proliferation was assessed by loss in fluorescence. **A,** representative dot plots **B,** cumulative data of Foxp3+ expression in cells at day 7 (top; n=5) and day 14 (bottom; n=3) **C,** representative histograms of CellTrace labelling in Foxp3- (red) and Foxp3+ (blue) populations **D,** cumulative data day 7 (top; n=5) and day 14 (bottom; n=3). **A,** differences in % Foxp3+ CD4+ T cells under different culture conditions was assessed by a paired t-test and **C,** differences in Foxp3+ and Foxp3- proliferation under different culture conditions were assessed by a paired t-test. * $p \leq 0.05$, **$p \leq 0.01$.

There is a significant increase in the frequency of Foxp3+ CD4+ T cells in cultures containing the combination of anti-IL-10R, TGFβ and 10^{-7}M $1,25(OH)_2D3$ at Day 7 and a similar trend is seen at Day 14 (Figure 5-16A and B). There was significantly more proliferation in the Foxp3+ versus the Foxp3- CD4+ cells after 7 and 14 Days of culture under these conditions.

5.3 Discussion

The data in this chapter demonstrate that high dose 1,25$(OH)_2$D3 (10^{-6}M) significantly increases the frequency of Foxp3+ CD4+ T cells in culture and this is associated with less inhibition of Foxp3+ CD4+ T cell proliferation as compared to Foxp3- T cells. The lower concentration of 1,25$(OH)_2$D3 (10^{-7}M), can increase the frequency of Foxp3+ T cells when it is in an environment high in TGFβ and/or low IL-10. Interestingly conditions that increased the frequency of Foxp3+ T cells i.e. 10^{-6}M 1,25$(OH)_2$D3 or 10^{-7}M 1,25$(OH)_2$D3 plus TGFβ, also had significantly more IL-2+ CD4+ T cells. There was enhanced phosphorylation of STAT5 in Foxp3+ Tregs in cultures containing 10^{-6}M 1,25$(OH)_2$D3 as compared to T effectors. Evidence for increased IL-2, CD25 and phosphorylation of STAT5, all point towards a mechanism where the Foxp3+ CD4+ T cells population may be better able to compete for available growth factor. A novel finding from this work was that 1,25$(OH)_2$D3 enhanced the gene expression of TGFβ as well as enhanced expression of LAP and GARP on the cell surface of CD4+ T cell in culture.

Together this data provides evidence for the following model:

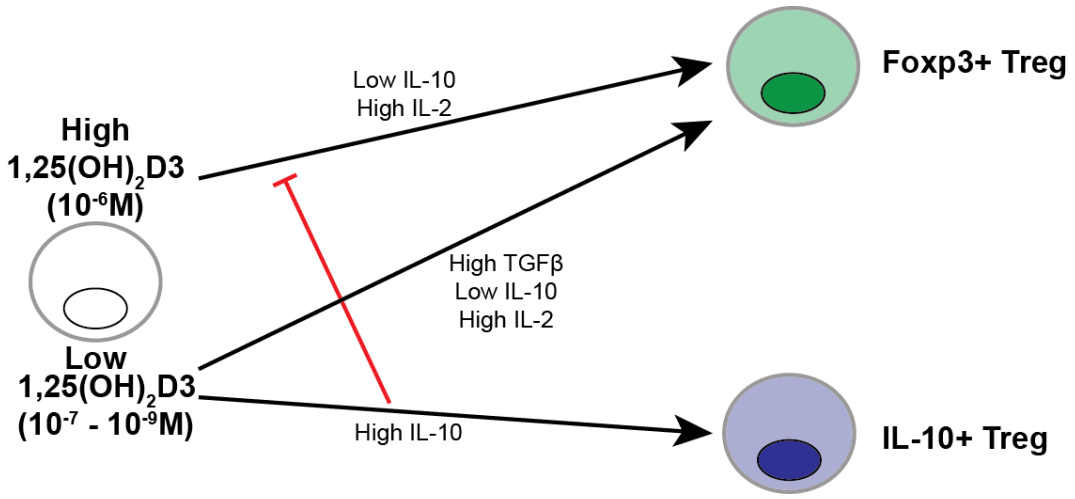


Figure 5-17 Proposed model for $1,25(OH)_2D3$ Treg generation

High concentrations of $1,25(OH)_2D3$ (10^{-6}M) in an environment low in IL-10 and high in IL-2 increases the frequency of Foxp3+ Tregs, whereas low concentrations of $1,25(OH)_2D3$ (10^{-7}M – 10^{-9}M) increase the frequency of IL-10+ Tregs. The cytokine milieu can also modulate Treg development as low concentrations of $1,25(OH)_2D3$ (10^{-7}M – 10^{-9}M) in combination with an environment low in IL-10 and high in IL-2 and TGFβ increases the frequency of Foxp3+ Tregs.

In this model low concentrations of $1,25(OH)_2D3$ (10^{-7}M – 10^{-9}M) increase the frequency of IL-10+ Tregs as reported from earlier studies from the lab [347,350,437], whereas high concentrations of $1,25(OH)_2D3$ (10^{-6}M) increase the frequency of Foxp3+ Tregs. Additionally the cytokine milieu can play an important role in the development of Tregs, as a high IL-10 environment inhibits Foxp3+ Treg development and promotes IL-10+ Tregs [347,350]. However if the $1,25(OH)_2D3$ environment is high in TGFβ and low in IL-10 then this increases the frequency of Foxp3+ Tregs [342].

Historically it was believed that the active form of vitamin D, $1,25(OH)_2D3$, was metabolised in the kidney, from the circulating precursor 25(OH)D. However subsequent work has identified that a number of cell types have the capability to generate $1,25(OH)_2D3$ from 25(OH)D, a number of which are found in the lung including APCs and epithelium [271-274]. These findings have identified that

monocyte-derived DCs can metabolise 1,25$(OH)_2$D3 to concentrations of $1x10^{-9}$M to $6x10^{-8}$M in culture [272,273,456]. Thus concentrations investigated in the work seem to be approaching physiologically relevant ones and certainly likely to represent concentrations potentially found in the lung, although it is still technically challenging to address this experimentally. It seems credible that concentrations of 1,25$(OH)_2$D3 required to increase IL-10 expression by CD4+ T cells can be achieved locally in the tissues, however whether the concentrations required to promote Foxp3 expression occur is less clear. However the role of 1,25$(OH)_2$D3 in promoting Foxp3+ Tregs in cultures is supported by my *ex vivo* data (Chapter 3 3-16) as well as earlier findings from our lab and other independent studies. These have shown that serum 25(OH)D positively correlates with the frequency and number of Foxp3+ CD4+ T cells in the peripheral blood and importantly with the frequency of lung Foxp3+ CD4+ T cells [312,342,395].

The cytokine environment has been shown to play an important role in Foxp3+ CD4+ T cell generation and my data implies that the cytokines IL-2, IL-10 and TGFβ have key functions in 1,25$(OH)_2$D3-induced Treg development. The addition of either anti-IL-10R or TGFβ in the presence of 10^{-7}M 1,25$(OH)_2$D3 modestly but significantly enhanced the frequency of Foxp3+ CD4+ T cells, however a combination of both was found to significantly increase the frequency of Foxp3+ CD4+ T cell *in vitro*. The proposed mechanism for the increase in Foxp3+ T cells was due to less profound inhibition of Foxp3+ CD4+ T cell proliferation by 1,25$(OH)_2$D3 at high concentration, as compared to that of Foxp3- CD4+ T cells. Additionally there was increased frequency of IL-2+ cells in conditions that increased the frequency of Foxp3+ T cells i.e. 10^{-6}M 1,25$(OH)_2$D3 or 10^{-7}M 1,25$(OH)_2$D3 and TGFβ. The data suggests that the Foxp3+ T cells were more efficient at utilising available IL-2 as shown by the enhanced phosphorylation of STAT5 in Foxp3+ Tregs as compared to T effectors. Evidence that

the Foxp3+ Tregs were out competing T effector cells in culture for the growth factor IL-2 was suggested since in the absence of Tregs there was more proliferation in the T effector cultures. Previously $1,25(OH)_2D3$ and TGFβ have been shown to inhibit IL-2 production from CD4+ T cells, in contrast to the data presented here where I see an enhancement of IL-2 [325,457]. However in those studies they investigated lower concentrations of $1,25(OH)_2D3$ and the TGFβ work was performed in a mouse model which may explain the differences observed. Nevertheless my data does provide a potential mechanism Foxp3+ T cells are increased in the presence of $1,25(OH)_2D3$ through production of IL-2, an essential cytokine for Treg function, maintenance, survival and stability of Foxp3+ cells [81,82,341,440,458-460].

Interestingly, $1,25(OH)_2D3$ was found to significantly increase the gene expression of *TGFβ1* and *TGFβ3*, as well as cell surface expression of GARP and LAP (surrogate markers of TGFβ expression) [447]. The role of $1,25(OH)_2D3$ on TGFβ is contrasting as $1,25(OH)_2D3$ has previously been shown to be important in increasing TGFβ expression from CD8+ and CD4+ T cells *in vitro*, whilst also locally controlling TGFβ expression within the skin [344,461,462]. A negative correlation between serum 25(OH)D and circulating levels of TGFβ has been reported, however 6 month supplementation with 25(OH)D has been shown to increase the levels of TGFβ secreted from PBMCs [463-465]. However to date there have been no publications demonstrating the effects of $1,25(OH)_2D3$ on LAP and GARP expression on CD4+ T cells.

The increased expression of LAP and GARP in the presence of 10^{-7}M $1,25(OH)_2D3$ predominantly occurred on the cell surface of Foxp3-. This was in contrast to earlier work which has shown that LAP and GARP were only expressed on the cell surface of Foxp3+ CD4+ T cells [451-453]. Since the optimum concentration for LAP and GARP induction was 10^{-7}M $1,25(OH)_2D3$ perhaps these cells are actually IL-10+ Tregs, and LAP and GARP may represent cell surface markers for these Tregs. Interestingly when

exogenous TGFβ is added into culture there was no significant effect on IL-10 gene expression. For optimum induction of Foxp3+ CD4+ T cells in cultures containing 10^{-7}M $1,25(OH)_2D3$ there was a requirement for exogenous, thus suggesting there may be additional functions of the $1,25(OH)_2D3$-induced TGFβ. TGFβ has been shown to be required for IL-10 production from human Tr1 cells *in vitro* and addition of an anti-TGFβ blocking antibody abrogated IL-10 production [466]. Clearly this is an area of work that would require additional research to fully comprehend what is the function of $1,25(OH)_2D3$-induced TGFβ.

Previous data by Jeffery *et al* found that $1,25(OH)_2D3$ had the capacity to convert naive CD4+ T cells into Foxp3+ Tregs in culture [341]. However previous work from our lab did not replicate this data, as the frequency of Foxp3+ T cells generated from naive CD4+ T cells was not increased in culture as compared to the no drug condition [342]. This is perhaps due to differences in signal strength in culture as Jeffery et al used a combination of anti-CD3 and CD28 coated beads, which provide a very robust stimulus, whereas we just used anti-CD3 and IL-2 [341,342]. The type of stimulation of the T cells is important as T cells need to be activated to upregulate VDR expression [467]. Additionally VDR has been shown to play a role in T cell receptor signalling [468]. VDR signalling has been shown to increase PLC-γ1 and cells were more responsive to TCR stimulation, thus differences in stimulations could impact on different Vitamin D responses [468].

Together this work provides further evidence for an important role of $1,25(OH)_2D3$ as an immunomodulatory molecule, as all concentrations of $1,25(OH)_2D3$ can promote Foxp3+ Tregs depending on the cytokine milieu. This data suggests that the administration of supplementation with vitamin D may represent an attractive therapy for enhancing endogenous populations of Tregs. Supplementation with vitamin D as a therapy to increase endogenous Treg populations is potentially a more practical, safer and may prove to be a cheaper method then those currently under development such

as Treg adoptive transfer in order to improve outcomes in transplantation an certain autoimmune conditions. Nevertheless ensuring vitamin D sufficiency in patients who have received Treg cell transfer might provide a conditioning environment that improves outcomes.

Therefore, 1,25$(OH)_2$D3 remains an attractive approach for treatment of chronic inflammatory diseases such as asthma, and vitamin D supplementation would be relatively safe, acceptable to patients and cost effective, and worthy of further investigation.

6. General Discussion

6.1 Conclusions

Asthma is a chronic inflammatory disease, characterised by airway remodelling, airway hyperresponsiveness and mucus hyperplasia. Asthma was originally described as a predominantly Th2-associated cytokine mediated disease, through effects of IL-4, IL-5, IL-9 and IL-13 on Th2-skewing of eosinophil, mast cell and B cell activation and antibody class switching to IgE. [165,166]. However asthma is in fact a heterogeneous disease and can present in many different phenotypes examples of which include: Th2-high, Th2-low, severe, neutrophilic and allergic asthma. These different asthma phenotypes present with similar symptoms albeit with different disease severity, yet the mechanism behind asthma development and exacerbations are different [165,166].

The current cornerstone treatment for asthma is glucocorticosteroids (steroids) and these work best in mild to moderate Th2-mediated asthma; these patients are known as steroid sensitive (SS) asthmatics [184,191]. A number of therapies are being developed as alternatives to steroids, and this is essential for the asthmatic phenotypes where steroids do not work [183]. Examples of such therapies include Pitrakinra (a competitive inhibitor of the IL-4Rα receptor) and Omalizumab (a humanised murine monoclonal antibody specific to the FcεR1-binding domain of IgE), but these therapies are specifically targeted to allergic asthmatics which is only a proportion of the asthmatic population [196,201,202]. Another Th2-specific therapy is Lebrikizumab (anti-IL-13 antibody) yet it was shown to be efficacious only in a population of asthmatics that had high serum levels of periostatin prior to treatment [168]. Thus these treatments although they demonstrate some clinical efficacy, are, however, extremely limited to certain phenotypes of asthma.

Severe asthma is defined as being uncontrolled asthma, with frequent severe exacerbations (or death) and/or failure to respond to medications and/or chronic morbidity [351] . Despite the treatment with high-dose of inhaled and/or oral steroids severe asthmatics have night and day symptoms, and low baseline lung function

(<80% predicted FEV_1) which limit their daily activities [167,351]. These patients report poor quality of life, and pose a huge economic and social burden upon the health service. Our clinical trial provided the exciting and unique opportunity to assess the phenotype of moderate/severe SS and SR asthmatics pre- and post-prednisolone to identify similarities and differences in the immune cell populations and their function.

Our lab has previously shown that CD4+ T cells from SR patients fail to produce IL-10 in response to steroids, in contrast to SS and healthy CD4+ T cells *in vitro* [349]. We have now shown additional immune cell differences in SR as compared to SS asthmatics, in particular Foxp3+ CD4+ T cells and dendritic cell (DC) populations. There were significantly fewer Foxp3+ CD4+ T cells in the peripheral blood of SR asthmatics as compared to SS. This observation may be linked to the lack of steroid responsiveness clinically, as independent studies suggest steroids enhance Foxp3+ Treg function, therefore fewer Foxp3+ Tregs could theoretically decrease the effectiveness of steroids [469]. There were significantly more mDCs in the peripheral blood of SR as compared to SS. These two data sets may be linked at there was a trend towards an increase in the ratio of CD11c/BDCA-4 DCs, and it has been previously been reported that pDCs (as defined by BDCA-4 expression) are involved in Treg induction. ILT3 has been implicated in the capacity of DC to enhance Treg experimentally, and evidence that ILT3 was expressed at much higher levels on pDCs then mDCs. Myeloid DCs have been implicated as being important for initiation and driving immune responses, and this is supported by the data showing that mDCs expressed higher CD86 and HLA-DR molecules on the cell surface. The differences seen in mDC and Foxp3+ CD4+ T cell in SS versus SR asthma populations could potentially be used as biomarkers for steroid responsiveness in severe asthma in conjunction with IL-17A expression.

The effect of a 2-week course of prednisolone on immune phenotype in SS and SR asthmatics was also investigated. There was a significant increase in the frequency

and number of CD19+ cells, which resulted in a significant change in the CD3+/CD19+ ratio. There was a significant reduction in the frequency of DCs, and a dramatic decrease in the pDC population. The reduction in pDCs post-steroids could have been due to a number of reasons including trafficking to the site of inflammation and/or that pDCs are susceptible to steroid-induced death [384]. A significant decrease in the frequency of Foxp3+ CD4+ T cells in the peripheral blood of SS and SR asthmatics post-steroids was also observed. This data differs from the earlier studies discussed above, and although the reasons for this are still unclear this could be due to differences in asthma severity since those earlier studies looked in the peripheral blood of adult patients with mild disease; it might also be due to recruitment of Foxp3+ CD4+ T cells into the lungs, as in asthmatic children this is where an increased frequency of Tregs was observed following inhaled steroids [172,174,188]. Interestingly although both SS and SR asthmatics had a similar frequency of CD4+ T cells pre- and post-steroids, there was a significant decrease in the number of CD4+ T cells in SS but not in SR. This is interesting as asthma is predominantly mediated by CD4+ T cells, thus a decrease in CD4+ T cell, which are potentially pathogenic, in SS asthmatics corresponds with clinical improvement in lung function. A big issue with asthma treatment is patient compliance, so a quick and informative way of measuring steroid compliance may be to look for a decrease in Foxp3+ CD4+ T cell and increase in CD19+ B cell frequency in the peripheral blood of asthmatics in combination with the current standard of measuring cortisol, although further studies are required to validate this. The aim of future transcriptional array analysis is to identify additional biomarkers of steroid compliance and response, which could be translated for use in the clinical to aid with diagnosis and treatment.

More recent epidemiological evidence suggests that severe asthma is partially mediated by Th17 cells and their associated cytokines IL-17A and IL-22. An elevated serum and BAL level of IL-17A appears to be both a marker of severe asthma as well

as for poor steroid responsiveness [370,371,404-408,426]. Our data provides additional evidence for a role of Th17 cells in severe asthma, as there is elevated secretion of Th17-associated cytokines from PBMC cultures from severe asthma patients as compared to healthy controls. Additionally when the severe asthmatics were further analysed according to steroid responsiveness clinically, there was 7-fold higher production of IL-17A from blood-PBMCs in SR donors as compared to the SS, but no significant difference between IL-22 was observed. Interesting, dexamethasone did not inhibit the IL-17A production and if anything seemed to enhance IL-17A production. This has important implications in the clinical setting as if these patients are not responding to steroids clinically further steroid use in these patients may actually be detrimental to these patients and may exacerbate disease. This data together with earlier epidemiological studies, suggests that IL-17A would provide a novel and exciting therapeutic target for severe asthma, however IL-17A has also been implicated in the clearance of mucosal infections, and this additional risk would need to be addressed. A number of anti-IL-17A monoclonal antibodies such as Secukinumab, are currently undergoing clinical trials for autoimmune diseases such as rheumatoid arthritis, psoriasis and Crohn's disease, and although the results to date have not all been positive they do provide an alternative potential therapy for severe asthma [470-473]. Perhaps a more targeted delivery of the anti-IL-17A therapy, i.e. in inhaler form, or even as discussed below, alternative therapies such as vitamin D, may be more efficacious in severe asthma.

Vitamin D sufficiency has been strongly associated with poor respiratory health [296,305]. There is increasing epidemiological evidence showing that Vitamin D insufficiency correlates with asthma development and severity [278,302]. Additionally low Vitamin D intake during pregnancy is associated with an increased incidence of wheeze, a surrogate marker for asthma in 2-5 year olds since asthma is generally not diagnosed before the age of six years [303,304]. Children and adults with low vitamin

D status are most likely to have severe asthma and are less likely to respond to asthma treatment [305-307]. The severe asthmatics in this clinical trial had much low levels of serum 25(OH)D as compared to healthy controls, and the SR patients had the lowest average levels of serum 25(OH)D. Even though these patients were recruited all year round there were only two patients out of the thirty-seven studied that had sufficient levels of serum 25(OH)D. The data observed in this clinical trial adds further support to accumulating evidence of the important link between Vitamin D insufficiency and asthma. The interest of our group lies in understanding immunological pathways that explain the link between low levels of serum 25(OH)D and immune mediated diseases with particular focus on asthma.

The active form of vitamin D, $1,25(OH)_2D3$, increases production of the anti-inflammatory cytokine IL-10 both directly and through enhancing glucocorticoid-induced IL-10 responses from CD4+ T cells *in vitro* [342,347,350,437]. IL-10 is an important anti-inflammatory cytokine in asthma, as polymorphisms in the IL-10 gene have been associated with asthma, resulting in lower production of IL-10 [223,226]. Additionally to the earlier data from our lab, $1,25(OH)_2D3$ has been shown to significantly decrease the expression of the pro-inflammatory cytokines IL-17A and IL-22, which we have shown are elevated in severe asthma. $1,25(OH)_2D3$-mediated inhibition of IL-17A is partially due to the increase in expression of the ectonucelotidase CD39, which has been shown previously to have the capacity to control Th17-mediated disease [138,140,419]. As discussed above targeting Th17/IL-17A directly in the airways and in asthma may be complicated by the fact that, based on data from animal models, it also plays an essential role in combating mucosal infections such as *Klebsiella pneumoniae*, and *Candida albicans* [398,399]. Concern exists, as an increased risk of infection has been observed with biologics targeting TNFα for other immune-mediated conditions [474,475]. Thus vitamin D, through its capacity to both inhibit IL-17A

production as well as enhance anti microbial pathways may offer a more attractive therapeutic strategy.

We and other have shown that 1,25$(OH)_2$D3 has the capacity to increase the frequency of Foxp3+ T regs and IL-10+ Tregs *in vitro* [341,342]. However 1,25$(OH)_2$D3 also increases the expression of a number of immunomodulatory pathways including CTLA-4, PD-1, CD200, CD39, CD73, LAP and GARP whilst downregulating GITR in CD4+ T cells in culture [341,342,434]. A body of evidence is emerging supporting this *in vitro* data, by showing a positive correlation *in vivo* in patients between serum 25(OH)D and the frequency or number of Foxp3+ T cells [312,395]. Additionally we have also shown that serum 25(OH)D, the accepted measure of vitamin D status, correlates with the frequency of Foxp3+ CD4+ T cells in the lungs of a cohort of paediatric severe asthma [342].

There is little co-expression of IL-10 and Foxp3 on the 1,25$(OH)_2$D3-induced Tregs, and this is in contrast to earlier mouse models which have shown that Foxp3+ Tregs express IL-10 however these Tregs were induced and analysed at mucosal sites, something which is difficult to study in humans [424,476]. Foxp3+ Treg function in mouse models of mucosal inflammation is inhibited through specifically knocking out IL-10 signalling in Foxp3+ Tregs [424]. Yet we have shown the opposite where inhibiting IL-10 signalling through the use of a blocking IL-10R antibody, actually enhances the frequency of Foxp3+ T cells *in vitro* [342]. Both the IL-10+ and Foxp3+ Tregs have been shown to have suppressive capacity, however when 1,25$(OH)_2$D3 is withdrawn from culture the Foxp3+ T cells are more stable than the IL-10+ T cells. Why 1,25$(OH)_2$D3 increases either IL-10+ or Foxp3+ is obviously instructed by the environment however it is assumed that they have different regulatory functions within the lung environment. It is likely that the phenotype of 1,25$(OH)_2$D3-induced Treg is dependent on the cytokine milieu, and we propose that the cytokines IL-2, IL-10 and TGFβ play an important role. It is unclear what the explanation is for the differences

observed in human and mouse studies with respect to co-expression of IL-10 and Foxp3. This could relate to relates to species differences however this seems unlikely since at least one human study has reported that 18% of Foxp3+ T cells also co-expressed IL-10 in the nasal mucosa following grass pollen allergen immunotherapy [477], thus co-expression can occur in humans at least at mucosal sites. Plasticity within T cell functional phenotypes and/or the transient mature of cytokine expression may also contribute, although further work is clearly required to provide a full understanding.

$1,25(OH)_2D3$-induced an increase in expression of LAP and GARP (surrogate markers of TGFβ) as well as CD39 and CD73 on Foxp3- and Foxp3+ CD4+ T cells. This was unexpected as LAP and GARP have been previously reported to only be expressed on Foxp3+ T cells [136-138,140,447,451,452], and this raises the possibility that these are markers of the IL-10+ Tregs. It would be extremely useful to have cells surface markers of IL-10+ Tregs, as IL-10 is difficult to detect *in vivo* without genetic manipulation of mice as well as directly *ex vivo* [478]. However, further work would need to be performed to identify if these cells are IL-10+ Tregs, and this would require sorting CD73/LAP+ versus CD73/LAP- populations and assesses the differences in IL-10 as well as total gene expression.

To conclude insufficiency in Vitamin D has been associated with a number of immune mediated diseases including asthma. We started these studies demonstrating that vitamin D has steroid-enhancing or sparing functions, and these data went on to form the basis of the clinical trial on which much of the present studies were based. We now show that vitamin D itself has a number of immunomodulatory properties including reducing proinflammatory cytokines and mediators such as ATP (through CD39 upregulation), IL-17A and IL-22; induction of anti-inflammatory cytokines and mediators

such as IL-10, TGFβ and Adenosine (through induction of CD39 and CD73) as well as directly inducing Foxp3+ Tregs. Together this provides further evidence to support further investigation of the use of vitamin D supplementation clinically, which we believe would enhance immunoregulatory pathways and would be particularly beneficial for the hard to treat therapy-resistant asthmatics.

6.2 Future Perspectives

6.2.1 SS versus SR gene arrays

As part of the clinical trial a number of cell pellets were collected (PBMCs, CD4+, CD8+ and CD8-depleted PBMCs) as well as whole blood directly stored and collected in Tempus Tubes from SS and SR asthmatics. These pellets are untouched and will provide an exciting opportunity to assess gene expression and methylation patterns, and compare SS and SR asthmatics pre- and post-steroids. This will hopefully enable the identification of biomarkers that could predict steroid responsiveness in the severe asthma cohort, with the potential to prevent unnecessary treatment of SR with high-dose steroids.

6.2.2 Does active Vitamin D restore response to steroids?

The main body of the clinical trial (Treatment Visits 1-3) is still blinded at the point of writing this report. Thus there is a massive body of data performed including direct ex vivo flow cytometry, 7-day culture in the presence or absence of drugs as well as the cell pellets stored at each clinical trial visit. With the unblinding of the clinical trial this will enable us to see primarily if there is an improvement in lung function of the SR asthmatics on calcitriol, in conjunction with analysing the immunological parameters as described before in Chapter 3 and 4. It will be very interesting to see what the effects of calcitriol are on Foxp3+ Treg populations, and particularly what happens to the

frequency of these cells post-steroids. Serum samples are also available at all time points. An important goal will be to identify biomarkers of disease status or phenotype as well as the likely response to treatment.

6.2.3 Future Clinical Trials

i. The current clinical trial was focussed on SR asthma, however there is emerging epidemiological evidence that vitamin D insufficiency is seen in all phenotypes of asthma, as highlighted by our own asthma patients. Additionally all moderate/severe patients would welcome the opportunity to reduce their steroid intake. Thus a clinical trial of vitamin D supplementation for all asthma patients would be of interest.

ii. The form of vitamin D used for treatment is still debated. In our clinical trial we used calcitriol, however this is suggested to have some negative feedback loop on vitamin D metabolism through upregulation of Cyp24a1, as well as increased risk of hypercalcemia. However there are individuals who have a reduced capacity to synthesize the active form of Vitamin D due to mutations in Cyp27B1, and these patients would perhaps benefit from treatment with calcitriol [294,295]. Therefore it would be interesting to perform a supplementation trial to allow comparison of the effects of calcitriol versus vitamin D3. The latter is likely to form a cheaper, safer option that has a much longer half-life *in vivo* (6 weeks compared to approx. 3 days) and may be more acceptable to patients.

iii. The lab is associated with other clinical trials including assessing the impact of vitamin D supplementation during pregnancy on development of wheeze in the offspring, and where parallel immunological analyses are in progress. A vitamin D supplementation trial in a cohort of severe therapy resistant paediatric asthma cohort is also planned.

6.2.4 Are CD73, LAP and GARP markers of IL-10+Tregs?

A recent paper identified that TGFβ plays an important role in induction of CD73 [427], thus it would be very interesting to see if vitamin D upregulation of CD73 expression can be inhibited by anti-TGFβ. CD73, GARP and LAP are all upregulated on Foxp3- CD4+ T cells and they are optimally upregulated in the presence of 10^{-7}M $1,25(OH)_2D3$, which suggests that these molecules could be cell surface markers of IL-10+ Tregs. This would need to be addressed by co-staining of CD73, LAP and GARP with intracellular IL-10. It would also be very informative to isolate the individual populations (CD73+LAP+) and to perform suppression assays as well as looking at the gene expression profiles of these cells.

6.2.5 Application of these findings into other immune-mediated disease

Steroid resistant disease is not restricted to asthmatic disease, as it has been shown in a number of autoimmune diseases including Inflammatory Bowel Disease, Multiple Sclerosis (MS) and Graft versus Host Disease [479,480]. MS would be a very interesting disease to investigate as there is a large body of evidence showing associations with Vitamin D insufficiency and MS development [289,290,294,295]. Although there are a number ongoing in MS with vitamin D supplementation (www.clinicaltrials.gov, accessed March 2013) there is not a trial focussed on supplementation of SR MS with Vitamin D, and I believe this would be extremely interesting. The overall aim of the MS trial would be to restore clinical response to steroids, through Vitamin D-dependent induction of Treg populations and decrease in inflammatory cytokine such as IL-17A, as we have seen experimentally and hope to confirm in our clinical trial.

Appendix I

Moderate to severe asthmatics were recruited from specialist asthma clinics at 3 different hospitals: Guys and St Thomas' NHS Foundation Trust, Barts and The London NHS Trust and Homerton University NHS Foundation Trust under the following inclusion and exclusion criteria:

Inclusion criteria:

1. Male or Female adults aged between 18 to 75 years.
2. Documented history and typical symptoms of asthma for ≥ 6 months prior to screening.
3. Pre-bronchodilator FEV_1 < 80% predicted and documented variability in airways obstruction of 12% or greater within the previous 5 years or diurnal Peak Flow variability of > 20%.
4. Corticosteroid refractory asthma, as defined by a < 10% improvement in FEV_1 following a 14 day course of prednisolone 40mg/1.73m^2/day[29-31].
5. Written informed consent received

Exclusion criteria:

1. Past or present disease, which, as judged by the investigator, may affect the study outcome (other than asthma, rhinitis or eczema).
2. Serum corrected calcium >2.66mmol/L
3. Clinically significant deviation from normal (physical examination or laboratory parameters) as judged by the investigator at the screening visit.
4. Current smoker or an ex-smoker of less than 5 years with a greater than 5 pack year history.
5. Pregnant or lactating females or those at risk of pregnancy (women of childbearing age may be offered a pregnancy test prior to recruitment).

6. History of a respiratory tract infection and/or exacerbation of asthma within 4 weeks of the screening visit requiring oral corticosteroid tablets.
7. Participation in a study involving an investigational medicinal product in the previous 3 months or blood donation within the last year.
8. Current or previous allergen immunotherapy.
9. Concomitant treatment with lithium carbonate or calcium supplements.
10. Inability to understand or comply with the research protocol

Subjects were withdrawn from investigational product treatment if they develop hypercalcaemia (corrected serum calcium >2.65 mmol/l confirmed on two samples) during the course of the trial. This was monitored at each follow-up time point.

Patients requiring rescue medication for exacerbation of asthma in form of corticosteroids immediately before and during the trial were excluded or withdrawn from the trial (this included the four week wash out period).

Throughout the study the participants were allowed to continue their usual asthma medication as prescribed by their doctor (for example short and long acting beta agonists (inhaled and oral form) including slow release, anticholinergica, inhaled corticosteroids, leukotriene receptor antagonists, theophyllines or antihistamines).

The severe asthmatics were given a 2-week course of oral prednisolone at 40mg/1.73m^2/day (where m^2 = Body Surface Area, BSA) at Screening Visit 1 and upon Screening Visit 2 they were assessed to identify Steroid Resistant Asthma. SR was defined as having less than 10% improvement in their lung function post a 2-week course of prednisolone as identified clinically by FEV_1. SS asthmatics (those who had >10% improvement in FEV_1) were excluded from the clinical trial (Treatment Visits 1 - 3). SR asthmatics were selected to enter the clinical trial after going through a 4-week washout. Upon entering the clinical trial the SR asthmatics were randomly and blindly assigned to either placebo or $1,25(OH)_2D3$ at Treatment Visit 1. After 2-weeks the

patients were given a further 2-weeks course of oral of prednisolone at the same dose at Screening Visit 1. Treatment Visit 3 was the final visit post-prednisolone. At all visits FEV_1 was assessed as the primary clinical readout. Furthermore exhaled Nitric Oxide was measured at Screening Visit 1 and 2 and Treatment visit 2 and 3 and Asthma Control Quality questionnaires were taken at Treatment visit 2 and Final visit to assess quality of life and asthma control. Along with this <100ml of peripheral blood was obtained to check for haematological and biochemical safety parameters throughout the study and cortisol levels to confirm compliance with prednisolone.

7.References

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8. Publications

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The Impact of Vitamin D on Regulatory T Cells

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Abstract Epidemiologic studies highlight the increasing prevalence of vitamin D deficiency and insufficiency and its association with an increased risk of autoimmune diseases and poor respiratory function, including asthma. These and additional studies have raised interest in the immunomodulatory properties of vitamin D beyond its well-established role in calcium homeostasis and bone health. Vitamin D has been shown to influence the function of cells intrinsic to innate and adaptive immunity. This review discusses recent evidence that vitamin D promotes—both directly and indirectly—regulatory or suppressor T-cell populations with the capacity to inhibit inappropriate immune responses that cause disease, suggesting that this property may in part underpin the epidemiologic findings.

Keywords Regulatory T cells · Interleukin-10 · FoxP3 · Vitamin D insufficiency · Tolerance

Introduction

Vitamin D

The active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1,25-[OH]2D3), is a secosteroid hormone that is mainly generated by a sunlight-catalyzed biosynthesis pathway that begins in the skin. UVB radiation (wavelengths, 270–300 nm) is absorbed by the epidermal and dermal cells and splits the B ring of 7-dehydrocholesterol, leading to the production of pre–vitamin D3. Pre–vitamin D3 is then rapidly converted to vitamin D3, which leaves the skin and enters the liver. In the liver, it is converted to 25-hydroxyvitamin D by cytochrome P450 enzymes (25-hydroxylases). 25-Hydroxyvitamin D is a circulating metabolite that is converted to the active form, 1,25-(OH)2D3, using the mitochondrial enzyme 25-hydroxyvitamin D-1 α-hydroxylase [1]. The conversion of 25-hydroxyvitamin D to 1,25-(OH)2D3 was originally thought to occur mainly in kidney cells, but there is increasing evidence of extrarenal sources of 1,25-(OH)2D3 facilitated by cells including macrophages, epithelial cells, and dendritic cells (DCs), which may represent an important source of vitamin D for immunomodulatory actions in tissues [1, 2, 3•].

1,25-(OH)2D3 binds to the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors; this leads to a conformational change in the VDR that results in binding to the retinoic X receptor (RXR) and formation of a heterodimer. This heterodimer translocates to the nucleus, where it can bind to a vitamin D response element and promotes transcription of vitamin D–responsive genes. The VDR–RXR heterodimer can also bind to a "negative" vitamin D response element and prevent gene transcription, or it can bind to transcription factors present in the nucleus and prevent binding to a target gene promoter [1]. VDR is expressed by many cells of the immune system, including activated B and T cells, monocytes, and DCs. This, together with epidemiologic evidence discussed subsequently, has aroused considerable interest in the immunomodulatory properties of vitamin D, including its capacity to promote regulatory T-cell (Treg) populations.

Vitamin D status is commonly measured by assessing the serum concentration of 25-hydroxyvitamin D. Although

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outside the scope of this review, there is ongoing discussion on which levels define deficiency, insufficiency, and sufficiency. However, vitamin D deficiency (<50 nmol/L) has been historically linked to bone conditions such as rickets and osteomalacia and is also important in osteoporosis and fracture risk [4]. More recently, widespread reports have identified that vitamin D insufficiency (between 50 and 75 nmol/L) is at levels thought likely to impact on immune function and is on the increase within the developed world due to changes in lifestyle during the past few decades. For example, a recent study demonstrated that during the winter and spring months, 87% of the United Kingdom population is vitamin D insufficient [2, 5]. However, vitamin D insufficiency is relatively common, even in equatorial regions in which lower levels of vitamin D in children were associated with increased markers of asthma severity [6]. Several studies have reported associations of low vitamin D status with poor pulmonary function and an increased incidence of respiratory infections, asthma, and chronic obstructive pulmonary disease [6–8].

Vitamin D sufficiency is associated with a reduced risk of colorectal and other cancers, while insufficiency has been associated with a range of autoimmune disorders, including multiple sclerosis, type 1 diabetes mellitus, inflammatory bowel disease, and rheumatoid arthritis [8]. These epidemiologic data highlighting the link between vitamin D insufficiency and a range of immune-mediated disorders have emerged in parallel with experimental studies on the immunomodulatory properties of vitamin D, in particular its capacity to inhibit effector T-cell responses and to promote Tregs, which may, at least in part, begin to explain some of these associations. Figure 1 is a schematic of mechanisms by which vitamin D promotes peripheral tolerance while maintaining innate immune mechanisms that are important for the defense against infection.

Regulatory T Cells

Several different Treg populations have been described with the capacity to inhibit a diverse array of immune responses and maintain immunologic tolerance in the periphery. The general belief is that Tregs exist in balance with effector T cells [9]. While effector T cells are essential for the elimination of many pathogens, they may also cause immune pathology through overexuberant or uncontrolled responses to pathogens, or inappropriate responses to self-antigens, commensal bacteria, and harmless environmental antigens such as allergens.

Although many cell types have the capacity to inhibit immune responses, most commonly via the production of immunosuppressive or anti-inflammatory mediators, considerable focus has been placed on T cells, particularly the $CD4^+$ T-cell subset, in part due to their capacity for antigen specificity. Natural Tregs that are selected in the thymus are thought to play an important role in maintaining peripheral tolerance and inhibiting immune responses to self-antigens. Additional mechanisms for the peripheral induction of Tregs are likely to be important to maintain the Treg population as the thymus involutes with age, and to generate Tregs with a wider array of antigen specificities.

The concept that T cells can suppress as well as enhance immune responses has been recognized for a considerable time. However, the "modern era" of Tregs emerged from studies by Sakaguchi and others in the mid-1990s [9, 10], as they described "natural" Tregs produced in the thymus. These cells were characterized by the constitutively high expression of the CD25 antigen, the α-chain of the interleukin (IL)-2 growth factor receptor. CD25 is normally only transiently expressed at moderate levels on effector T cells upon activation. Further work identified—first in mice and subsequently confirmed in humans—that the lineage-specific transcription factor for these cells was forkhead box P3 (FoxP3) [11]. Nevertheless, identification of cell surface antigens that uniquely distinguish Tregs from T effector cells or differentiate between different Treg populations has proven elusive. "Naturally" occurring $FoxP3^+$ Tregs are vitally important for self-tolerance and immune homeostasis, as best highlighted by cases of immune dysregulation polyendocrinopathy, enteropathy X-linked syndrome (IPEX), in which patients lack Tregs due to mutations in the gene *FOXP3*. IPEX is a fatal condition due to widespread autoimmune, allergic, and inflammatory disease from a very early age unless it is treated aggressively by bone marrow transplantation or profound immune suppression. A similar condition, scurfy, has been described in mice [11].

Adaptive Tregs are generated in the periphery via several different experimental protocols often associated with suboptimal activation of immune responses, such as via immature or alternatively activated antigen-presenting cell (APC) populations [12]. The capacity of effector T cells to deviate toward a Treg phenotype is also increasingly being described [13]. Adaptive or induced Tregs comprise both $FoxP3^+$ and $FoxP3^-$ populations and synthesize anti-inflammatory mediators such as IL-10, transforming growth factor (TGF)-β, IL-35, and adenosine. The various Treg populations may additionally control immune responses through mechanisms including inhibitory cell surface ligands such as cytotoxic T-lymphocyte antigen (CTLA)-4 and programmed cell death 1 ligand 1 (PD-L1), cytotoxic mediators such as granzyme A and B, competition for growth factors, or via metabolic disruption involving CD39 and CD73 [14]. The range of inhibitory mechanisms used by Tregs has been the subject of recent excellent reviews [14].

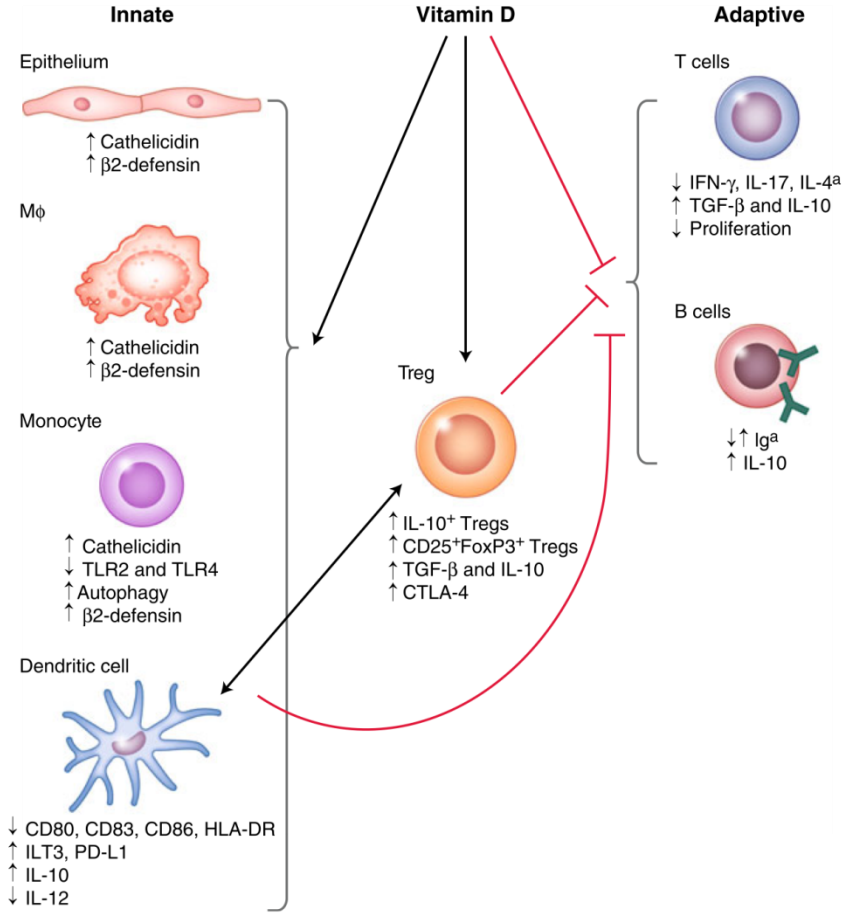

Fig. 1 Effects of vitamin D on cells of the innate and adaptive immune response likely to promote peripheral tolerance while maintaining innate immune mechanisms important for the defense against infection. Vitamin D promotes an antimicrobial environment via induction of antimicrobial peptides such as cathelicidin and β2-defensin. In conjunction with this, vitamin D renders dendritic cells (DCs) more immature via downregulation of costimulatory molecules *HLA-DR*, CD86, CD80, and the maturation marker CD83, and inhibition of the T-helper type 1 (Th1)-skewing cytokine interleukin (IL)-12. It also induces expression of inhibitory ligands such as immunoglobulin-like transcript 3 (ILT3) and programmed cell death 1 ligand 1 (PD-L1), and the anti-inflammatory cytokine IL-10. DCs are rendered tolerogenic and promote $FoxP3^+$ and $IL\text{-}10^+$ regulatory T cells (Tregs). Vitamin D also acts directly on T cells to promote $FoxP3^+$ and $IL\text{-}10^+$ Tregs, secretion of the immunomodulatory cytokines IL-10 and transforming growth factor (TGF)-β, and upregulation of the inhibitory molecule cytotoxic T-lymphocyte antigen (CTLA)-4. These Tregs subsequently inhibit innate and adaptive immune responses by downregulating proinflammatory cytokines, upregulating IL-10 and TGF-β, and via effects on a range of cell types, including antigen-presenting cells and T cells. [a]There is a lack of consensus on the effects of vitamin D on Th2 and IgE allergic-type responses, but recent data may provide an explanation demonstrating significant but nonlinear effects of vitamin D on these parameters. IFN—interferon; TLR—Toll-like receptor

Effects of Vitamin D on Effector T-cell Responses and Antigen-Presenting Cell Function

The magnitude and nature of the T-cell response is dependent on the context in which antigen is presented to T cells by dedicated APCs. DCs in particular play a central role in this process and are viewed as a pivotal link between innate and adaptive immunity. Innate pattern-recognition receptors expressed by DCs trigger their activation and are central for skewing the adaptive immune responses that are subsequently induced. Additional local environmental signals, such as inflammatory cytokines and in all likelihood 1,25-(OH)2D3, influence DC phenotype and activation and thus the nature of the T-cell response (eg, T-helper type 1 [Th1], Th2, Th17, Treg). Conversely, immature DCs with poor stimulatory function for the induction of effector T-cell responses often drive Treg responses.

Most of the direct immunomodulatory properties of 1,25-(OH)2D3 on DCs are proposed to occur in myeloid (mDCs) and not plasmacytoid DCs (pDCs). A study by Penna et al. [15] suggests that although both primary human blood–derived mDCs and pDCs express comparable levels of VDR and upregulate the primary response gene *CYP24* upon culture with 1,25-(OH)2D3, only the tolerogenic properties of mDCs are modulated upon culture with 1,25-(OH)2D3. A recent study of gene profiling of vitamin

D–treated DCs suggests that 1,25-(OH)2D3 upregulates several target genes that render the DCs more immature. These data identify complex patterns of gene expression via microarray analysis. Importantly, these data have been validated in part on primary human peripheral blood mDCs [16].

In functional studies, 1,25-(OH)2D3 treatment of human DCs in vitro results in reduced expression of the costimulatory molecules CD80 and CD86 and decreased expression of HLA-DR and the maturation marker CD83, all associated with an immature DC phenotype. 1,25(OH)2D3 also affects DC maturation from monocytes through inhibition of the production of IL-12 p70 and enhanced IL-10 secretion upon activation by CD40 ligation [17, 18]. 1,25-(OH)2D3 induces expression of immunoglobulin-like transcript 3 (ILT3) in vitro, an inhibitory receptor containing three cytoplasmic immunoreceptor tyrosine-based inhibitory receptor motifs, which when activated by the ligand result in an intracellular inhibitory signaling cascade [19]. The relevance of this in vitro observation is highlighted by studies in patients with psoriasis, in whom topical treatment of their lesions with 1,25-(OH)2D3 resulted in increased expression of ILT3 on DCs in conjunction with a reduction in the histology score of the lesions [19]. In vitro, 1,25-(OH)2D3-induced ILT3 appears to play a role in inhibiting T-cell responsiveness, as blockade of ILT3 on 1,25-(OH)2D3-treated DCs resulted in increased production of interferon (IFN)-γ [19]. In addition, upon activation, monocyte-derived DCs treated with 1,25-(OH)2D3 express enhanced levels of PD-L1, blockade of which significantly increased IFN-γ secretion in T-cell co-cultures [20].

Vitamin D inhibits effector T-cell responses via modulation of APC function and via direct inhibition of T-cell responses. The active form of vitamin D (1,25-[OH]2D3) decreases the production of the autocrine T-cell growth factor IL-2 via inhibition of the transcription factor known as nuclear factor of activated T cells, and this leads to a marked reduction in $CD4^+$ T-cell proliferation [21]. 1,25-(OH)2D3 inhibits Th1 cytokine release with a large reduction in the production of IFN-γ from human peripheral blood $CD3^+CD4^+$ T cells [22]. 1,25-(OH)2D3 also hinders cytokine production by Th17 cells in murine autoimmune disease models, as well as in human $CD4^+$ T cells in vitro [23, 24].

The effects of vitamin D on Th2 responses are less clear, with reports of both inhibition and enhancement. Early murine model data, including the use of a VDR knockout mouse, suggested that 1,25-(OH)2D3 enhanced the development of Th2 cells [25, 26]. A study using human peripheral blood mononuclear cells demonstrated that active vitamin D at a single very high dose enhanced Th2 cytokine production [27]. However, other studies in naive murine and human T cells reported that 1,25-(OH)2D3 inhibited the expression of IL-4 and other Th2 cytokines in vitro [28, 29]. It is notable that in animal models in which an increased Th2 response was observed [23, 25], this was accompanied by an increase in IL-10, symptomatic of a regulatory rather than an inflammatory Th2 phenotype [30]. Results of two recent human studies, one assessing serum and vitamin D status IgE [31•] and a second looking at a broad dose response of active vitamin D on human $CD4^+$ T cells in culture [32], suggest a nonlinear effect of vitamin D on Th2 responses. These reports may help explain the earlier divergent data, suggesting overall that only abnormally low or high levels of vitamin D will adversely influence Th2 responses. Of additional interest here, Kreindler et al. [33] recently reported that in patients with allergic bronchopulmonary aspergillosis, heightened Th2 reactivity, assessed as serum IL-5 levels, correlated with low vitamin D status [33]. Similarly, ingestion of calcitriol (the active form of vitamin D, 1,25-[OH]2D3) by steroid-refractory asthma patients failed to increase Th2 cytokine gene expression in $CD3^+CD4^+$ T cells analyzed directly ex vivo but did increase IL-10 gene expression [32]. These data suggest, together with other studies discussed subsequently, that concomitant with the inhibition of an effector response is the induction of tolerance and Treg populations.

Vitamin D and Regulatory T Cells

A range of experimental approaches have been used to highlight the capacity of vitamin D to modulate $FoxP3^+$ and $IL\text{-}10^+$ Treg numbers and/or function. These range from in vitro and in vivo murine models, to patient-based studies demonstrating associations between vitamin D status and Treg numbers and/or function, and studies assessing Treg status before and after delivery of vitamin D to patients through supplementation with the parent compound or pharmacologic delivery of the active moiety. Together, these data have generated considerable interest in the therapeutic application of vitamin D to enhance Treg function from early life through to adulthood in a wide range of immune-mediated disorders.

Human In Vitro Studies

Inhibition of effector or inflammatory responses is frequently paralleled by the induction of a tolerogenic response and of Tregs. In this context, several studies have shown that pretreatment of human blood–derived mDCs, both monocyte-derived and primary mDCs, with 1,25-(OH)2D3 and then co-cultured with T cells not only inhibited T-effector cytokine production but also induced $CD4^+FoxP3^+$ cells with suppressive activity [19, 34•]. However, ILT3 expression induced by 1,25-(OH)2D3 on DCs in culture appeared to be dispensable for the capacity of 1,25-(OH)2D3-treated DCs to

induce $CD4^+FoxP3^+$ Tregs, which is in contrast to its proposed role in modulation of the Th1 cytokine response [19].

1,25-(OH)2D3 may additionally act directly on T cells to promote a Treg phenotype. In bulk cultures of human $CD4^+CD25^-$ T cells and putative naïve T cells, 1,25-(OH)2D3 was reported to increase the frequency of activation-induced $FoxP3^+$ T cells, and this was dependent on the presence of IL-2 in culture. These $FoxP3^+$ Tregs expressed high levels of CTLA-4, an inhibitory receptor. A significant reduction in the proinflammatory cytokines IFN-γ and IL-17 was also observed [35]. However, a comparable study using naïve murine T cells suggests that 1,25-(OH)2D3 inhibits both IL-17 and Treg differentiation in vitro [36].

A significant body of literature describes the capacity of retinoic acid to induce $FoxP3^+$ Tregs, originally stemming from studies in murine gut [37]. Retinoic acid and vitamin D are members of the same nuclear hormone family and share a common signaling receptor, RXR [1]. It is presently unclear whether retinoic acid and vitamin D play complementary or redundant roles in Treg induction, or whether they play a comparable role in mice and humans. Furthermore, an emerging concept is the plasticity within the T-cell lineage and the capacity of previously activated effector T cells and differentiated T-cell lineages to convert to a regulatory phenotype [13]. Thus, it also will be of interest to determine, for example, whether vitamin D and retinoic acid target the same T-cell population (eg, naive vs previously activated T cells). Finally, differential effects of these two mediators on T-cell homing, referenced below, may indicate different roles in distinct mucosal compartments.

Vitamin D positively influences the IL-10–secreting Treg populations. Unger et al. [20] demonstrated that pretreatment with 1,25-(OH)2D3 upregulates expression of PD-L1, an inhibitory receptor, on monocyte-derived DCs and that these modified DCs were able, upon co-culture, to convert $CD4^+$ T cells into IL-10–secreting Tregs capable of suppressing the proliferation of responder T cells [20]. In our own studies, activation of human and murine $CD3^+CD4^+$ T cells in vitro in the presence of a combination of the glucocorticosteroid dexamethasone (Dex) and 1,25-(OH)2D3 induced a sizeable population of IL-10–secreting Tregs. The regulatory capacity of these IL-10–secreting Tregs generated in vitro was assessed in vivo in a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Transfer of the $IL\text{-}10^+$ Tregs resulted in prevention of EAE in this model. The cells equally inhibited human Th1 and Th2 responses in vitro, and this was reversible through inhibition of IL-10 signaling in culture [38].

Subsequent studies demonstrated that human peripheral blood $CD4^+$ T cells polyclonally activated in the presence of 1,25-(OH)2D3 alone also promoted IL-10–secreting Tregs. The microbial pattern-recognition receptor known as Toll-like receptor (TLR)9 was described as a biomarker of vitamin D–induced $IL\text{-}10^+$ Tregs, and ligation of TLR9 with its agonist CpG oligonucleotide turned off IL-10 production, suggesting a control mechanism whereby Treg function may be abrogated [32]. This may be of relevance, for example, during infection, in which the capacity to transiently block inhibitory function would facilitate a more effective immune response for clearance of the pathogen. Elimination of the pathogen would lead to diminished TLR ligation, allowing restoration of the Treg response and thereby minimizing tissue damage [39]. Expression of microbial pattern-recognition receptors is more commonly associated with cells of the innate immune response. Schauber et al. [40] found that 1,25-(OH)2D3 acted upon keratinocytes to increase expression of the microbial pattern-recognition receptors TLR2 and CD14 and upregulate cathelicidin. The authors proposed that vitamin D acts in innate immunity also, enabling keratinocytes to recognize and respond to microbes and to protect wounds against infection. Thus, vitamin D deficiency may be important in the predisposition of skin from patients with atopic dermatitis to superinfection by *Staphylococcus aureus* [40].

The capacity of 1,25-(OH)2D3 to induce IL-10 and TLR9 on human $CD4^+$ T cells was also demonstrated directly in patients. $CD3^+CD4^+$ T cells were analyzed directly ex vivo from steroid-refractory asthma patients before and after calcitriol treatment at standard formulary doses. Increased *IL-10* and *TLR9* gene expression was observed following calcitriol ingestion [32]. Earlier work on human cord blood determined that samples obtained during the summer months (hence higher levels of serum 25-hydroxyvitamin D) had a significantly higher level of IL-10 compared with samples obtained during winter months with lower serum vitamin D [41], further supporting the observation that vitamin D increases IL-10 in vivo.

These studies were shown to have clear application to human disease, specifically asthma. Earlier observations demonstrated that steroids induce IL-10 synthesis via human $CD4^+$ T cells from healthy donors and steroid-sensitive asthma patients, but not in asthma patients who failed to give a good clinical response to steroids for improvement of their lung function—termed *steroid insensitive* or *refractory*—suggesting that the induction of IL-10 might contribute to the clinical efficacy of steroids in asthma [42]. The culture of peripheral blood T cells from steroid-refractory asthma patients with both Dex and active vitamin D completely restored the defective steroid-induced IL-10 response to levels observed in cultures of cells from steroid-sensitive asthmatics that were cultured with Dex alone. More strikingly, ingestion of oral 1,25-(OH)2D3 (calcitriol) by steroid-refractory asthmatics restored the capacity of the T cells to produce IL-10 in vitro in response to Dex. Mechanistic studies suggested that 1,25-(OH)2D3 influenced downregulation of the glucocorticoid receptor

by its ligand [43]. These studies suggest that vitamin D may have therapeutic potential in severe asthma patients as a steroid-enhancing agent and are complemented by emerging studies showing an association between low vitamin D status and poor clinical responsiveness to glucocorticoids in asthma patients [6, 44]. If a steroid-enhancing role for vitamin D is proven, this is likely to be applicable to other chronic illness in which steroids represent a primary treatment, as also suggested by animal model data [23].

In conjunction with 1,25-(OH)2D3 effects on T cells, a recent article showed that 1,25-(OH)2D3 treatment of human B cells enhanced production of the immunomodulatory cytokine IL-10 [45]. 1,25-(OH)2D3 may therefore have an additional regulatory effect on B cells, potentially converting B cells into cells with a more regulatory role, although these concepts are still preliminary.

Animal Model Studies

Animal models have provided a wealth of data, largely in agreement with human laboratory studies, on the capacity of vitamin D to promote Tregs. Although subtle differences exist in the data from the varying models, in general, these demonstrate that irrespective of the route of administration, vitamin D mediates the expansion of Tregs and amelioration of immune-mediated diseases in vivo. In vitro and in vivo data provide evidence for promotion of tolerogenic DCs and enhanced IL-10, TGF-β, FoxP3, and CTLA-4 expression.

Early murine studies demonstrated that 1,25-(OH)2D3 alone or in combination with immunosuppressive drugs enhanced $CD25^+FoxP3^+$ Tregs in vivo. In a transplant model, a combination of oral mycophenolate mofetil (selective T- and B-cell inhibitor) and 1,25-(OH)2D3 prior to and during transplantation resulted in acceptance of and tolerance to a fully mismatched mouse islet allograft [46]. This tolerance was associated with decreased DC expression of CD40, CD80, CD86 and IL-12; reduced Th1 cytokine production; and an increased frequency of $CD25^+CD4^+CD152^+$ Tregs, which could transfer transplant tolerance to a recipient mouse.

Vitamin D has beneficial effects in many animal models of autoimmune disease, including antiretinal autoimmunity [24], acute colitis [23], diabetes [47], arthritis [48], and EAE [34•, 36]. In a study of the progression of diabetes in nonobese diabetic mice, 1,25-(OH)2D3 alone inhibited Th1 infiltration and the progression of diabetes associated with an increased frequency of $CD4^+CD25^+$ Tregs in pancreatic lymph nodes [47]. In an EAE model, oral administration of active vitamin D prevented disease onset and $CD4^+$ T cells in the central nervous system and decreased $IL\text{-}17^+$ cells in the spleen and central nervous system, albeit with no change in IL-10 or IFN-γ and a slight decrease in $FoxP3^+$ T cells in the spleen. In vitro, the authors described increased IL-10 production but inhibition of FoxP3 by vitamin D due to the inhibition of IL-2 required for $FoxP3^-$ Treg induction [36]. In an acute colitis model, intraperitoneal administration of calcitriol alone, or more effectively with Dex, reduced the severity of disease and local Th1 parameters. Locally increased IL-10, TGF-β, FoxP3, and CTLA-4 were all reported. The authors concluded that their data support a steroid-sparing clinical application for calcitriol derivatives in inflammatory bowel disease [23].

Two independent groups suggest that topical administration of 1,25-(OH)2D3 to the skin of mice resulted in the in vivo expansion of antigen-specific $CD25^+CD4^+FoxP3^+$ Tregs in the skin-draining lymph node [49, 50]. These Tregs demonstrated an enhanced capacity to suppress immune responses in vitro and in vivo, and these data are clearly relevant to the topical use of active vitamin D in psoriasis [19].

In contrast, some early studies suggested that vitamin D has deleterious effects in allergic airway disease. VDR-deficient mice failed to develop experimental allergic asthma, leading the authors to suggest a role for vitamin D in driving Th2 inflammation in the airways [26]. Nevertheless, considerable interest remains in the therapeutic application in asthma, and examples of beneficial effects exist [51]. In a murine model of allergic airway disease, 1,25-(OH)2D3 potentiated the effects of a suboptimal allergen (ovalbumin) immunotherapy regimen to ameliorate airway hyperresponsiveness and to reduce eosinophilia, Th2 responses, and IgE (in serum) via induction of the regulatory cytokines IL-10 and TGF-β [52]. When human $CD4^+$ T cells obtained from allergic bronchopulmonary aspergillosis (Th2-mediated disease) patients were cultured with the addition of 1,25-(OH)2D3, reduced DC expression of OX40L and increased TGF-β expression by DCs and Tregs were reported, together with inhibition of Th2 responses [33].

1,25-(OH)2D3 not only influences the generation of tolerogenic immune responses but also expression of chemokines and their receptors that are likely to determine T-cell homing. For example, 1,25-(OH)2D3 signaled T cells to express CC chemokine receptor (CCR)10, which enabled them to migrate to the skin-specific chemokine CCL27 secreted by keratinocytes of the epidermis but suppressed the gut-homing receptors α4β7 and CCR9, a property more commonly associated with retinoic acid [53]. 1,25-(OH)2D3 inhibits the expression of CCL17 and CCL20 but induces expression of CCL22 in mDCs that are suggested to play a key role in the recruitment of Tregs [15]. Clearly defining the effects of vitamin D on T-cell homing is an important component in our understanding of its importance in the regulation of immunity.

Therapeutic Application of Vitamin D in Humans: Evidence for Immunomodulatory Actions

Epidemiologic studies highlighting associations with vitamin D status and various immune disorders have heightened

interest in addressing further correlations with immunoregulatory cytokines and Treg activity in patients. For example, recent evidence shows that in multiple sclerosis patients, low serum 25-hydroxyvitamin D status (but not serum 1,25-dihydroxyvitamin D3) was associated with reduced suppressive capacity of $CD25^{+}CD4^{+}CD127^{lo}$ Tregs [54]. Similar associations have been shown for IL-10 [41]. In humans, vitamin D supplementation and pharmacologic treatment with active vitamin D have been shown to increase serum- or T-cell–associated TGF-β and IL-10, respectively [32, 55].

Conclusions

The active form of vitamin D (1,25-[OH]2D3) influences innate and adaptive immunity. It acts on APCs and T cells to promote peripheral tolerance via inhibition of inflammatory responses and induction of Tregs. Growing interest exists in the physiologic role of vitamin D as an essential mediator in maintaining a healthy and functional immune system. However, many questions remain regarding its role compared with other related compounds such as retinoic acid. Therapeutic evaluation of vitamin D supplementation and active vitamin D, including analogues with reduced calcemic effects, is ongoing. Studies of this nature will be crucial to furthering our understanding of both the physiologic role of vitamin D in the maintenance of health and its therapeutic evaluation, including steroid-enhancing activity, for a range of immune and inflammatory disorders.

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impact of B-cell immunodeficiency in cases of PNP deficiency and is probably explained by the complete lack of PNP enzyme activity due to the reported nonsense mutation. Numerous symptomatic mutations, mostly missense, of the *PNP* gene have been identified. These mutations produce proteins with variable degrees of enzymatic activity, which correlate with the accumulation of nucleoside substrates and, to some degree, with the clinical course. It is the progressive accumulation of deoxyguanosine triphosphate in PNP deficiency that leads to progressive disease. Interestingly, similar to the progressive T-cells toxicity caused by PNP deficiency during the first years of life, the more severe B-cell defect observed in our older patient also suggests accumulating effects of PNP deficiency on B cells. Our results concur with several recent *in vitro* and *in vivo* studies describing B-cell death following PNP inhibition with excess deoxyguanosine/deoxyguanosine triphosphate concentrations, albeit at a reduced frequency compared with T-cell apoptosis.[11] Additional studies, including longitudinal studies, are still required to determine whether B-cell function, like T-cell function, deteriorates over time in PNP-deficient patients. In normal peripheral blood B cells, the addition of deoxyguanosine leads to an inhibition of proliferation and differentiation. This effect was found to be independent of deoxyguanosine accumulation.[12] Complete lack of PNP (as observed in our patients) triggers accumulation of deoxyguanosine, thereby disrupting B-cell development, the consequence of which is more profound with time, as indeed was found in the older sister. Another line supporting the hypothesis that the accumulation of deoxyguanosine results in global cell toxicity and that it is not restricted to T cells alone came from the use of purine and pyrimidine nucleoside analogs to treat certain malignancies, including B-cell malignancies.[13] For example, forodesine, a PNP inhibitor, converts deoxyguanine to deoxyguanosine triphosphate and causes general apoptotic cell effect, similar to what is seen in PNP deficiency.[14]

We conclude that the variable effect on B-cell function that is observed in PNP deficiency may be an independent event of T-cell dysfunction and that it is subject to the severity of the deficiency and the duration of the disease.

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Serum 25-dihydroxyvitamin D levels correlate with CD4$^+$Foxp3$^+$ T-cell numbers in moderate/severe asthma

To the Editor:

Asthma is a chronic inflammatory disease characterized by airways hyperresponsiveness, mucus hyperplasia, and airways remodeling. The immune and inflammatory processes that underpin asthmatic disease are complex and include evidence for impaired immune regulation by both Foxp3$^+$ and IL-10$^+$ regulatory T (Treg) cells (reviewed in Lloyd and Hawrylowicz[1]).

The major treatment for asthma is corticosteroids (steroids), enabling most asthmatic patients to achieve some control of their symptoms (steroid sensitive [SS]). However, a proportion of asthmatic patients fail to gain any clinical benefit in terms of lung function and are termed steroid resistant (SR), representing those most at risk from their asthma. Corticosteroids enhance the production of the antiinflammatory cytokine IL-10 by T cells *in vitro*, but notably this response is impaired in cultures from SR asthmatic patients, implying an association between impaired IL-10 response and poor asthma control.[2] This defect in steroid-induced IL-10 can be restored by the addition of the active form of vitamin D_3 (1,25-hydroxyvitamin D_3) into the culture, thus suggesting that vitamin D may play a role in controlling steroid responsiveness.[3] These data are complemented by a number of independent clinical studies that highlight a high prevalence

TABLE I. Patient characteristics

	Steroid sensitive	Steroid resistant
Age (y)	50 (21-64)	53 (30-67)
Sex ratio (female/male)	4/4	5/9
FEV_1 (L)	1.8 (1.33-2.21)	2.06 (1.17-2.65)
FEV_1 (L) poststeroids	2.29 (1.77-3.08)	1.99 (1.06-2.53)
FEV_1 (% predicted)	61.6% (45-76)	65.6% (40-79)
FEV_1 (%) poststeroids	77.6% (68-87)	64.6% (36-80)
FENO	26.5 (18-42)	27.6 (12-70)
Atopy (yes/no)	7/1	12/2
Race		
Caucasian	6 (75%)	9 (64%)
Afro-Caribbean	2 (25%)	4 (29%)
Asian	0 (0%)	1 (7%)
Average inhaled corticosteroid dose (BDP equivalent) (μg)	1225 (800-2000)	1228 (800-2000)
Serum 25(OH)D	37.54 (20-91)	40.63 (27-57)

Data shown as mean and range.
BDP, Beclometasone dipropionate; *FENO*, fraction of exhaled nitric oxide.

of vitamin D deficiency and insufficiency worldwide and its association with an increased incidence, severity, and poor control of asthma.[4,5]

The importance and status of a well-defined subset of Treg cells, as defined by the expression of the transcription factor forkhead box P3 (Foxp3), in SS and SR asthma are less well understood.[1,6] Although 1,25-hydroxyvitamin D_3 has been shown to enhance the frequency of human $Foxp3^+$ Treg cells *in vitro*, no *in vivo* correlates of these data exist.[7] The aim of the present study was therefore to investigate whether differences exist in the frequency of $Foxp3^+$ Treg cells in the peripheral blood of SS and SR adult asthma patients, and the relationship between vitamin D status and the $Foxp3^+$ Treg-cell compartment.

Adults aged 18 to 75 years with moderate to severe asthma for at least 6 months on therapy step 3 or 4 of the British Thoracic Society guidelines on management of asthma, and who remained clinically poorly controlled, were recruited. All patients had a prebronchodilator FEV_1 of less than 80% of the predicted value with reversibility of more than 12% following 400 μg of short-acting bronchodilator and had undergone detailed assessment to exclude an incorrect diagnosis and comorbidities affecting asthma control. SR asthma was defined as an increase of less than 10% in FEV_1 following a 2-week course of prednisolone of 40 mg/1.73 m^2 body surface area. Smokers and participants who had suffered from a respiratory tract infection or asthma exacerbation during or 4 weeks prior to enrolling for the study were excluded. This study was approved by the NHS research ethics committee London Bridge (08/H0804/84), and all patients gave written informed consent. Fourteen SR and 8 SS patients with mean ages of 54 and 50 years, respectively, were assessed. Mean prebronchodilator FEV_1 was 2.06 L (65.6%) and 1.99 L (64.6%) in the SR patients and 1.80 L (61.6%) and 2.29 L (77.6%) in the SS patients before and after the course of oral corticosteroid, respectively (poststeroid FEV_1 SR vs SS: $P < .001$; 95% CI for difference 0.27-0.78 L; analysis of covariance with presteroid FEV_1 as covariate). The patients did not significantly differ in their mean body mass index (28.6 vs 31.1), inhaled corticosteroid dose (beclometasone dipropionate equivalent: 1228 μg vs 1225 μg), or race (9 Caucasian, 5 Afro-Caribbean/Asian origin vs 6 Caucasian, 2 Afro-Caribbean origin) as can be seen in Table I.

Flow cytometry was performed on peripheral blood from each patient obtained pre–oral corticosteroid. Treg cells were defined as $Foxp3^+$ cells within the $CD4^+CD3^+$ lymphocyte population as shown in Fig 1. Additional staining demonstrated that between 90% and 95% of the $Foxp3^+$ cells were $CD127^{lo}CD25^{hi}$, markers that distinguish Treg cells from recently activated effector T cells.[8] SR asthma patients had a significantly lower frequency of $Foxp3^+$ T cells as compared to SS asthma patients at baseline (mean SR, 7.18%; mean SS, 9.77%; $P = .015$). Serum 25-hydroxyvitamin D (25(OH)D) concentrations were assessed by using a 2-dimensional high performance liquid chromatography system—tandem mass spectrometry.[9] Although patients were recruited all year round, all but 1 patient was vitamin D insufficient (<75 nmol/L). No correlation was observed between body mass index and serum 25(OH)D or inhaled steroid dose and serum 25(OH)D. There were significantly lower levels of serum 25(OH)D in patients who were of Afro-Caribbean origin than in Caucasians (mean Caucasian origin 38.55 nmol/L vs Afro-Caribbean/Asian origin 23.13 nmol/L; $P = .045$). There was also a trend toward seasonal differences observed, with highest levels of serum 25(OH)D seen in the summer and autumn and lowest levels seen in the winter and spring, but this did not achieve statistical significance.

A strong positive correlation between serum 25(OH)D levels and $Foxp3^+$ T-cell number in all asthmatic patients was observed (Pearson correlation $r = 0.7$; $P = .0003$).

This cross-sectional study demonstrates that the frequency of circulating $Foxp3^+$ $CD4^+$ Treg cells is significantly lower in steroid resistant than in SS asthmatic patients with comparable disease severity. A majority of patients with severe asthma had serum concentrations of 25(OH)D within the deficient range, and this strongly correlated with a paucity of $Foxp3^+$ Treg cells in the peripheral blood. These data suggest a strong functional correlation between vitamin D status, assessed as circulating 25(OH)D, circulating $CD4^+Foxp3^+$ Treg-cell numbers, and corticosteroid responsiveness in chronic asthma. They also provide important *in vivo* validation of *in vitro* studies with human cells and evidence from animal models suggesting that the vitamin D pathway plays an important role in enhancing the frequency of the $CD4^+Foxp3^+$ Treg-cell compartment in humans.

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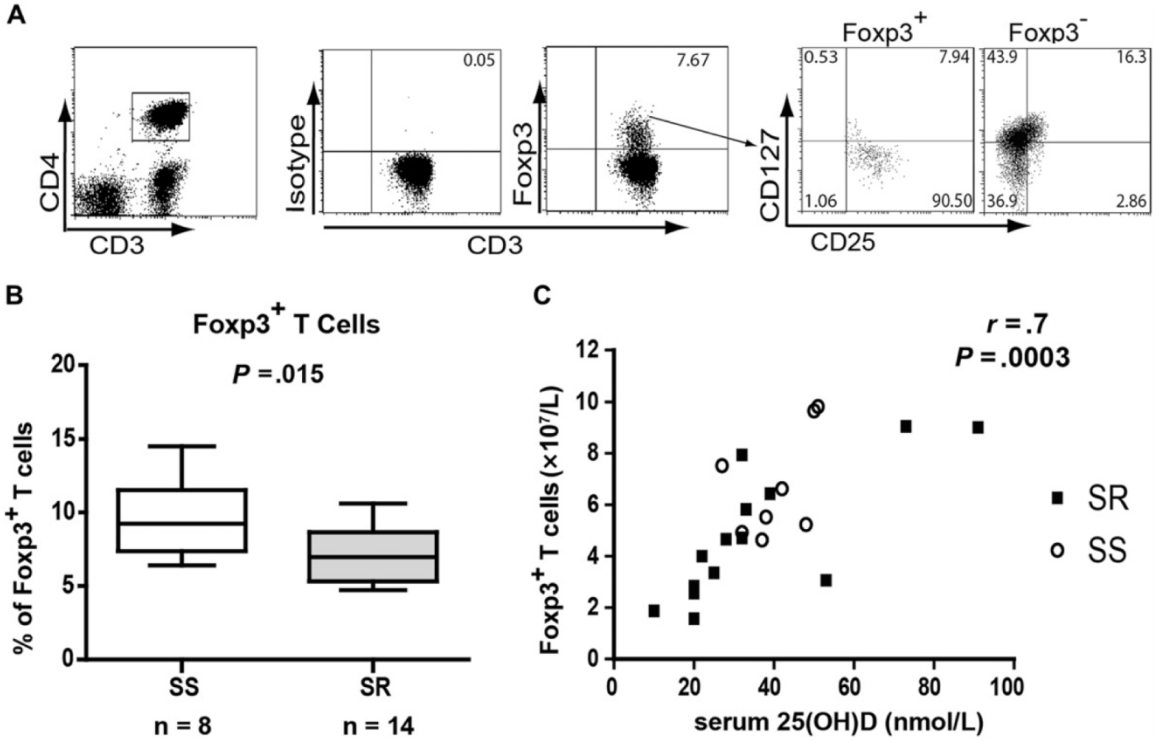

FIG 1. SR asthmatic patients have a lower frequency of Foxp3$^+$ Treg cells than do SS asthmatic patients, and serum 25(OH)D concentration correlates positively with Foxp3$^+$ Treg cells in the peripheral blood. **A,** Representative dot plots demonstrating the gating strategy to define Treg cells. Values represent % of gated live CD4$^+$CD3$^+$ lymphocyte population. **B,** Frequency of Foxp3$^+$ Treg cells in SS and SR asthmatic patients. Data shown as mean, 5%-95% CI, assessed by *t* test. **C,** Correlation of Foxp3$^+$ Treg cells with serum 25(OH)D in all the patients with moderate to severe asthma. Assessed by Pearson correlation test.

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The broad spectrum of interepithelial junctions in skin and lung

To the Editor:

Asthma and atopic dermatitis represent the biggest group of chronic noncontagious diseases in children and adults with the largest burden on health care costs. The better understanding of the molecular mechanisms has opened a way for the development of many novel treatment modalities.[1,2] Two recent articles published in the *Journal of Allergy and Clinical Immunology* demonstrated the disruption of epithelial barrier function of keratinocytes in the skin of patients with atopic dermatitis and bronchial epithelial cells in the lungs of asthmatic patients.[3,4] These studies suggest that tissue integrity is disturbed in patients, and allergens, bacterial toxins, and other particles are able to penetrate the epidermis and the lung epithelium, where they may activate the immune system leading to severe chronic inflammation in both diseases. Therefore, paracellular sealing of keratinocytes and bronchial epithelial cells appears to be very important to prevent the infiltration of the dermis and submucosa by factors that induce allergic inflammation.

There are several types of cell-cell adhesion and sealing complexes in between epithelial cells: tight junctions (TJs), adherens junctions, gap junctions, and desmosomes (Fig 1, *A*). Main contributors to the barrier function of epithelia are TJs. These form the most apical cell-cell adhesion complexes regulating the paracellular flux of water and ions as well as of larger compounds.[5] They are able to regulate the apical-basolateral

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The role of 1α,25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3$^+$and IL-10$^+$ CD4$^+$ T cells

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1α,25-Dihydroxyvitamin D3 (1α25VitD3) has potent immunomodulatory properties. We have previously demonstrated that 1α25VitD3 promotes human and murine IL-10-secreting CD4$^+$ T cells. Because of the clinical relevance of this observation, we characterized these cells further and investigated their relationship with Foxp3$^+$ regulatory T (Treg) cells. 1α25VitD3 increased the frequency of both Foxp3$^+$ and IL-10$^+$ CD4$^+$T cells in vitro. However, Foxp3 was increased at high concentrations of 1α25VitD3 and IL-10 at more moderate levels, with little coexpression of these molecules. The Foxp3$^+$ and IL-10$^+$ T-cell populations showed comparable suppressive activity. We demonstrate that the enhancement of Foxp3 expression by 1α25VitD3 is impaired by IL-10. 1α25VitD3 enables the selective expansion of Foxp3$^+$ Treg cells over their Foxp3$^-$ T-cell counterparts. Equally, 1α25VitD3 maintains Foxp3$^+$ expression by sorted populations of human and murine Treg cells upon in vitro culture. A positive in vivo correlation between vitamin D status and CD4$^+$Foxp3$^+$ T cells in the airways was observed in a severe pediatric asthma cohort, supporting the in vitro observations. In summary, we provide evidence that 1α25VitD3 enhances the frequency of both IL-10$^+$ and Foxp3$^+$ Treg cells. In a translational setting, these data suggest that 1α25VitD3, over a broad concentration range, will be effective in enhancing the frequency of Treg cells.

Keywords: 1α,25-Dihydroxyvitamin D3 · Asthma · Immune regulation · Regulatory T cells

Supporting Information available online

Introduction

Considerable interest exists in the therapeutic potential of regulatory T (Treg) cells to treat a range of immune-mediated pathologies in humans. This is partly based on evidence obtained from animal models of human disease demonstrating the capacity of Treg cells to control transplant rejection, and to successfully treat autoimmune and allergic disease [1]. Two broad therapeutic

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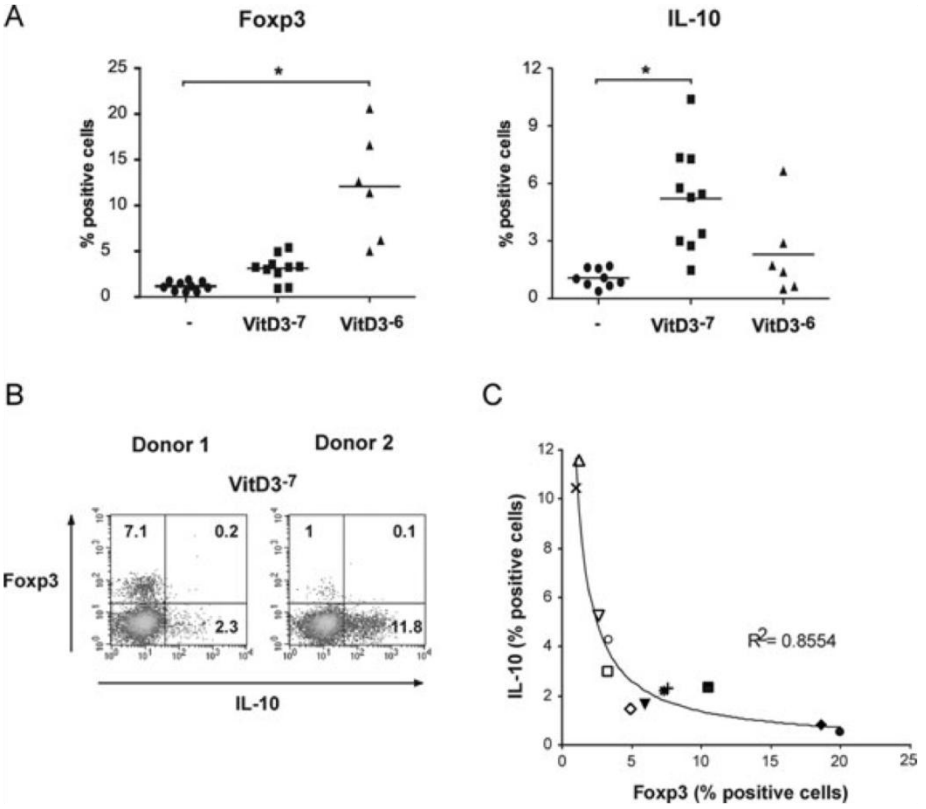


Figure 1. 1α25VitD3 increases the frequency of IL-10$^+$ and Foxp3$^+$ human CD4$^+$ T cells. Human CD4$^+$ T cells were stimulated for two 7-day cycles with anti-CD3, IL-2, and IL-4 (No VitD3) or additionally with the indicated concentration of 1α25VitD3 (VitD3; 10× M). (A) At day 14, cells were restimulated for 16 h with anti-CD3 and IL-2. IL-10$^+$ cells were identified using an IL-10 secretion assay kit. FoxP3$^+$ cells were assessed by intranuclear staining. Values represent the percentage of gated live CD4$^+$ cells. Each symbol represents an individual donor and lines represent the mean. $^*p < 0.05$ as determined by the Mann–Whitney rank sum test. (B) Cells were costained for expression of both IL-10 and FoxP3 in the presence of 10^{-7} M 1α25VitD3. Two representative flow cytometry plots from different donors are shown. Note the absence of FoxP3$^+$IL-10$^+$ cells. Data are representative of seven independent experiments. (C) Data from the costaining experiments are depicted in a correlation analysis. R^2 value was determined by Spearman's rank correlation coefficient. Each symbol represents a different donor ($n = 8$); closed symbols = 10^{-6} M 1α25VitD3, open symbols = 10^{-7} M 1α25VitD3.

strategies are being considered in research initiatives worldwide: (i) adoptively transferring Treg cells that have previously been expanded in vitro into patients and (ii) inducing or boosting endogenous Treg cells directly in patients. The latter approach may be more applicable in highly prevalent conditions such as allergy and asthma, which cause considerable morbidity, but are generally not life threatening. The rationale for such a strategy is further strengthened by evidence that existing therapies for allergic diseases, such as allergen immunotherapy and glucocorticoids, are associated with the induction of Treg cells in patients [2]. Nevertheless, considerable scope for improving the safety and efficacy of these treatments exists.

Recent studies have focused on the capacity of vitamin D to modulate Treg-cell subsets. For example, culturing dendritic cells (DCs) with the active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α25VitD3) leads to impaired DC maturation, development of tolerogenic properties [3], and the capacity to induce CD4$^+$Foxp3$^+$ cells with suppressive activity [4], or IL-10 expressing Treg cells [5]. In animal models of human disease, administration of 1α25VitD3 successfully treats transplant rejection [6] and a range of autoimmune conditions, including antiretinal autoimmunity [7], acute colitis [8], diabetes [6], arthritis [9], and EAE [10], as well as allergic airway disease [11]. These studies demonstrate a correlation between therapeutic efficacy and increased frequency or quantities of CD4$^+$CD25$^+$ T cells, IL-10, TGF-β, and CTLA-4.

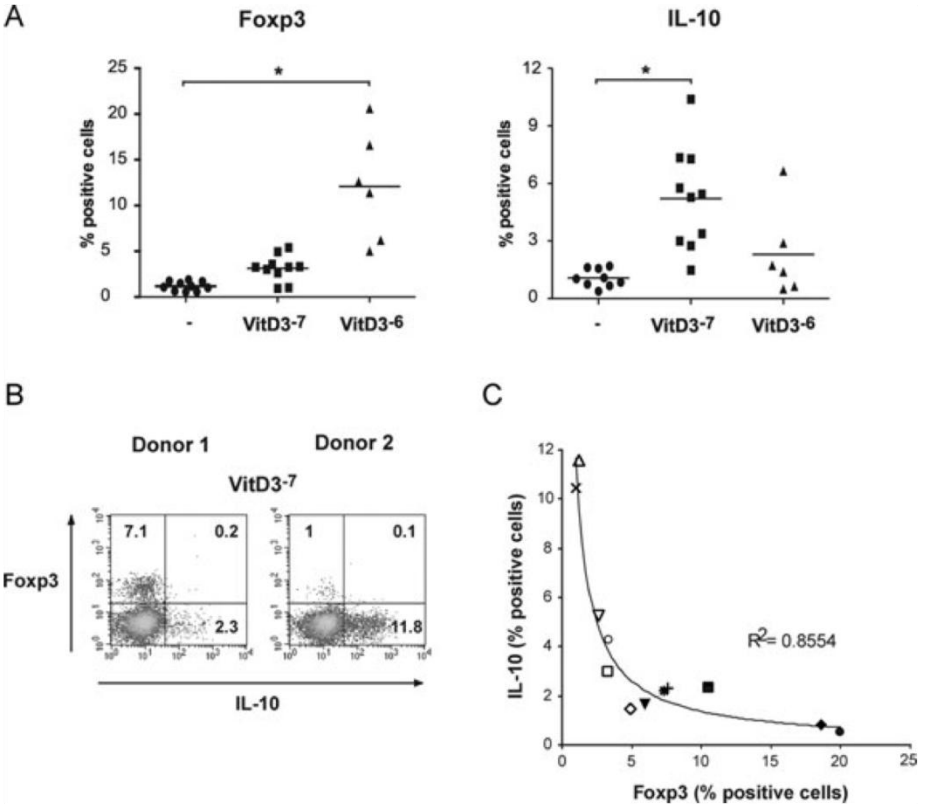
Our earlier studies have highlighted the capacity of 1α25VitD3 to promote human CD4$^+$ IL-10 secreting Treg cells (IL-10-Treg) in culture both alone [12] and in concert with glucocorticoids such as dexamethasone [13, 14]. Furthermore, treatment of severe steroid refractory asthma patients with 1α25VitD3 in vivo directly increased IL-10 gene expression in CD3$^+$CD4$^+$ T cells [12], and restored the impaired steroid-induced IL-10 response in CD4$^+$ cells in vitro [14, 15].

The present study was designed to further investigate the mechanisms underlying the therapeutic potential of 1α25VitD3 in the context of asthmatic disease, and to determine effects on the induction of both IL-10$^+$ and Foxp3$^+$ T cells. Specifically, we have examined the effects of 1α25VitD3 on total, unfractionated CD4$^+$ T-cell populations, representative of those likely to be encountered in vivo. The data demonstrate that 1α25VitD3 increases the frequency not only of IL-10-Treg cells, but also of Foxp3$^+$ Treg cells, that these cells express increased levels of the inhibitory receptors CTLA-4 and PD-1, and exhibit inhibitory function. The data further suggest that 1α25VitD3 functions to maintain Foxp3 expression in the existing Foxp3$^+$ Treg-cell pool.

Results

1α25VitD3 increases the frequency of both IL-10$^+$ and Foxp3$^+$ human CD4$^+$ T cells in culture

We have previously described the induction of IL-10 secreting cells following culture of human CD4$^+$ T cells with 1α25VitD3 in vitro and directly ex vivo following administration of calcitriol to



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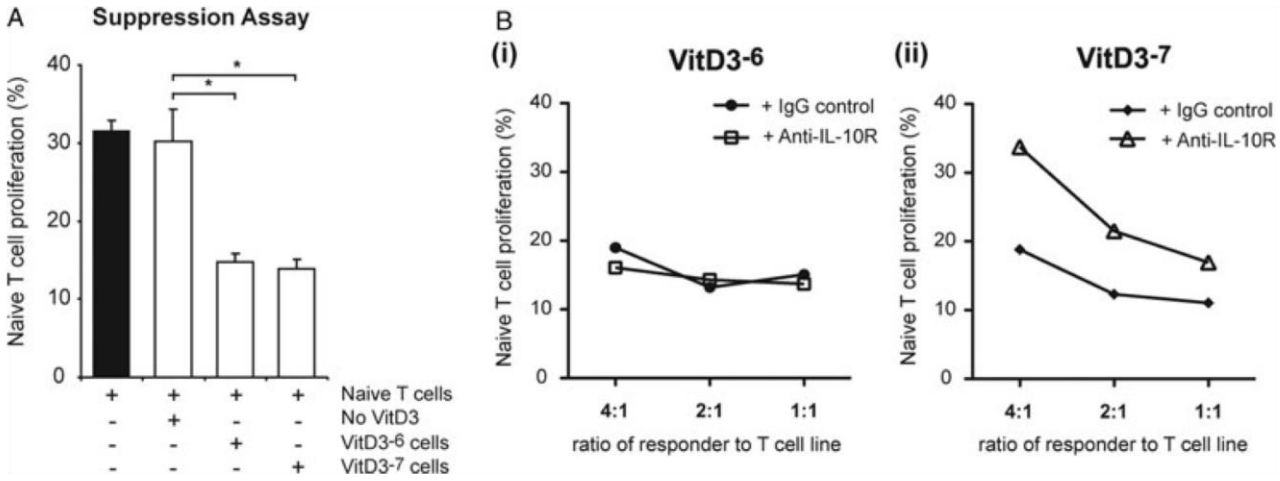

Figure 2. 1α25VitD3-treated CD4$^+$ T-cell populations acquire suppressive properties. (A) Autologous CD45RA$^+$ T cells were isolated, CFSE-labeled, and co-cultured with the cell lines (No VitD3 or VitD3, as indicated) at a ratio of 2:1 responder to cell line, for 5 days with anti-CD3 and CD28. The percentage of proliferating, viable CFSE-labeled responders is shown, as assessed by flow cytometry. Data are shown as mean ± SEM from four independent experiments from different healthy donors. *$p < 0.05$ as determined by the Mann–Whitney rank sum test. (B) CFSE-labeled responder cells were co-cultured with cell lines — (i) VitD3 10^{-6}M T cells, (ii) VitD3 10^{-7}M T cells–at the ratios indicated in the graph, in the presence of control IgG (closed symbols) or anti-IL-10R (hollow symbols); both at 5 μg/mL. Data are representative of four independent experiments.

asthma patients [12, 14]. An unusual dose response was observed in vitro with 1α25VitD3 at the very highest concentration tested (10^{-6} M 1α25VitD3) resulting in considerably lower IL-10 secretion than the optimal concentrations of 10^{-7} M and 10^{-8} M 1α25VitD3 [12]. Here, we analyzed this dose response further and investigated whether IL-10 was being synthesized by Foxp3 positive or negative T cells in response to 1α25VitD3 in culture.

Human peripheral blood CD4$^+$ T cells were stimulated with anti-CD3, IL-2, and IL-4 under conditions previously determined to optimally induce IL-10-Treg cells [12]. The expression of Foxp3 and IL-10 in the presence or absence of 1α25VitD3 was determined by flow cytometry. 1α25VitD3 at 10^{-6} M led to an increase in Foxp3 expression as compared with control cultures (No VitD3), whereas lower doses of 1α25VitD3 minimally affected Foxp3 expression. In contrast, maximal IL-10 induction was observed, as expected, at 10^{-7} M and 10^{-8} M 1α25VitD3 [12]. This response was confirmed using a panel of donors. A statistically significant increase in the frequency of Foxp3$^+$ T cells was observed at 10^{-6} M, but not at 10^{-7} M 1α25VitD3, while significant induction of IL-10$^+$ T cells was seen at 10^{-7} M, but not at 10^{-6} M (Fig. 1A). In summary, 1α25VitD3 enhances both the percentage of Foxp3$^+$ cells and the expression of Foxp3 transcripts (data not shown), but at a distinct concentration of 1α25VitD3 than required for optimal IL-10 induction.

In our early studies, cells were analyzed for expression of Foxp3 and IL-10 independently by flow cytometry. To confirm and extend the finding of differential effects of 1α25VitD3 on these molecules, a protocol for costaining was developed. CD4$^+$ T cells were cultured with 10^{-6} M or 10^{-7} M 1α25VitD3 and then restimulated with anti-CD3 and IL-2 for 16 h and analyzed for expression of IL-10 and Foxp3 by secretion assay and then intranuclear staining.

In two representative donors, shown in Figure 1B, virtually no (≤0.2%) cells stained positive for both Foxp3 and IL-10 in the presence of 10^{-7} M 1α25VitD3. When cells from a panel of healthy donors were screened, we observed that cell cultures preferentially expressed a high frequency of Foxp3$^+$ cells and low levels of IL-10, or conversely low Foxp3 and a high frequency of IL-10$^+$ cells in response to culture with 1α25VitD3 (Fig. 1B and C).

Since Foxp3 expression may not always reflect inhibitory function, the functional consequences of 1α25VitD3 modulation of Foxp3 versus IL-10 expression by human CD4$^+$ T cells was next investigated.

1α25VitD3-enhanced IL-10 and Foxp3$^+$ T cells show regulatory function and phenotype

CD4$^+$ T-cell lines generated from the same donor in the presence of either high (10^{-6} M; Foxp3-promoting Treg conditions) or lower (10^{-7} M; IL-10-Treg favoring conditions) concentrations of 1α25VitD3 were tested for their capacity to inhibit the proliferation of autologous, naïve CFSE-labeled responder T cells. Both populations showed comparable inhibitory activity (Fig. 2A). The suppression by cells generated with 10^{-7} M 1α25VitD3 could be diminished by the addition of anti-IL-10 receptor antibody to the co-culture, while in T-cell cultures generated with 10^{-6} M 1α25VitD3, the antibody had little effect (Fig. 2B), suggesting both IL-10-dependent and IL-10-independent mechanisms of suppression existed in the two different populations.

To further investigate the phenotype of 1α25VitD3-treated cells, T cells were cultured with a range of concentrations (10^{-9}–10^{-6} M) of 1α25VitD3 and analyzed for expression of surface markers by flow cytometry. A number of Treg-associated molecules, including the inhibitory molecules PD-1 and CTLA-4, as well as CD38 and CD25 were shown to be increased following exposure to 1α25VitD3, although the expression of the Treg-associated marker, GITR, and also CD62L, were inhibited by 1α25VitD3 (Fig. 3).

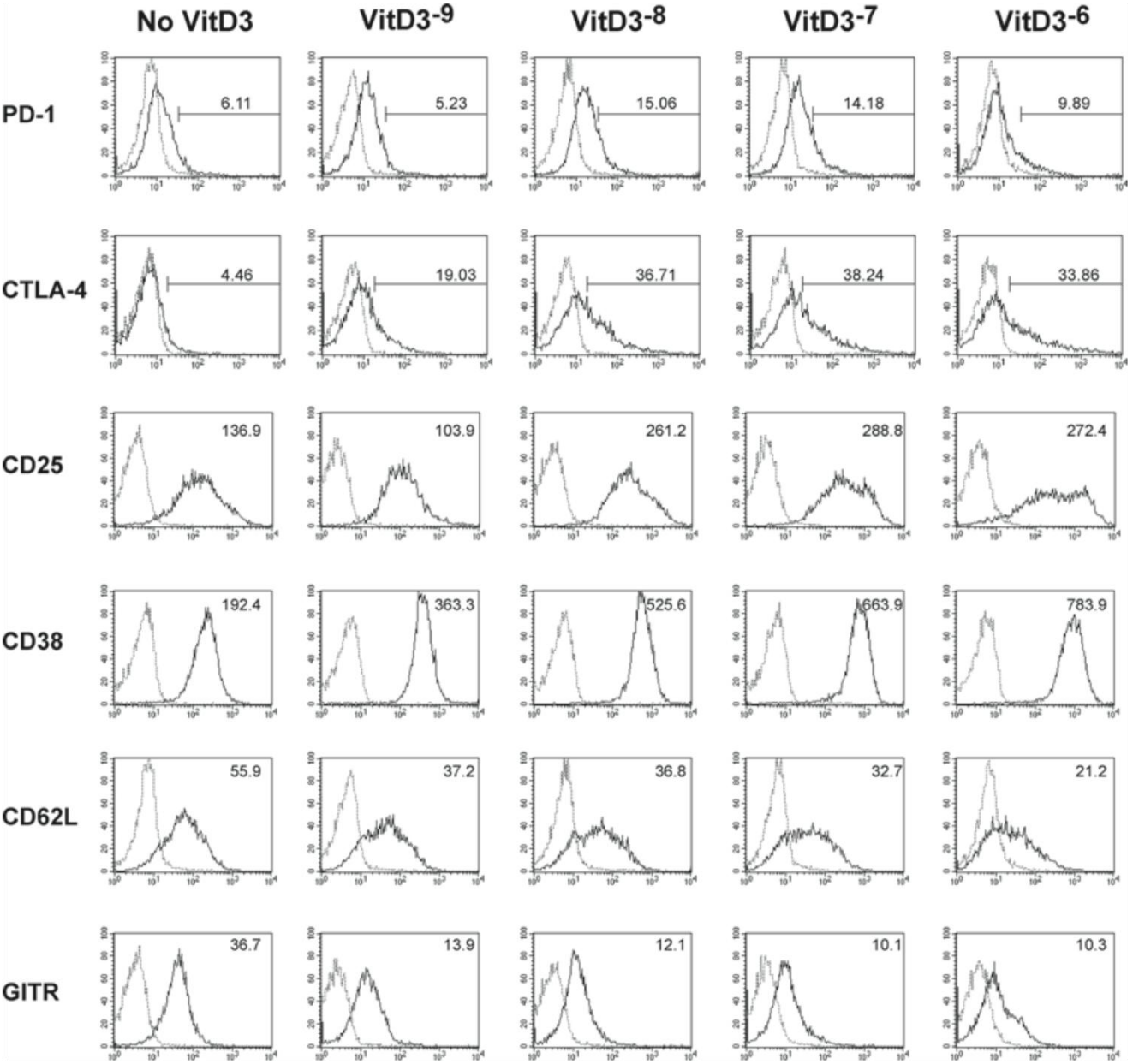

Figure 3. 1α25VitD3 promotes the expression of Treg-cell-associated surface markers. Human $CD4^+$ T cells were stimulated alone (No VitD3) or additionally in the presence of the indicated molar concentrations of 1α25VitD3 (VitD3). At day 14, cells were stained and analyzed for the surface expression of CD25, CD38, PD-1, CTLA-4, CD62L, and GITR (black lines; gray lines = matched isotype control) by flow cytometry. For PD-1 and CTLA-4, values represent percentage of positive cells; all other antigens values shown are indicative of the geometric mean fluorescence intensity. Data are representative of a minimum of six independent experiments.

The enhancement of Foxp3 expression by 1α25VitD3 is impaired by IL-10

We have previously shown that IL-10 expression is reduced when IL-10 signaling is neutralized in culture [12, 13]. Cells were stimulated in the absence or presence of 10^{-8} –10^{-6} M 1α25VitD3 together with either an anti-IL-10R antibody or the appropriate isotype control reagent. In a representative donor shown in Fig. 4A, a high frequency of $Foxp3^+$ cells was observed following culture with 10^{-6} M 1α25VitD3 and the presence of anti-IL-10R antibody in culture did not alter this. In contrast, considerably less $Foxp3^+$ cells were detected in cell cultures containing 10^{-7} M or 10^{-8} M 1α25VitD3, and the addition of anti-IL-10R to these cultures resulted in a marked increase in the frequency of $Foxp3^+$ cells (Fig. 4A; mean data from four healthy donors depicted in Fig. 4C). These data were also replicated at the mRNA level using real time RT-PCR where addition of anti-IL-10R antibody resulted in a significant increase in

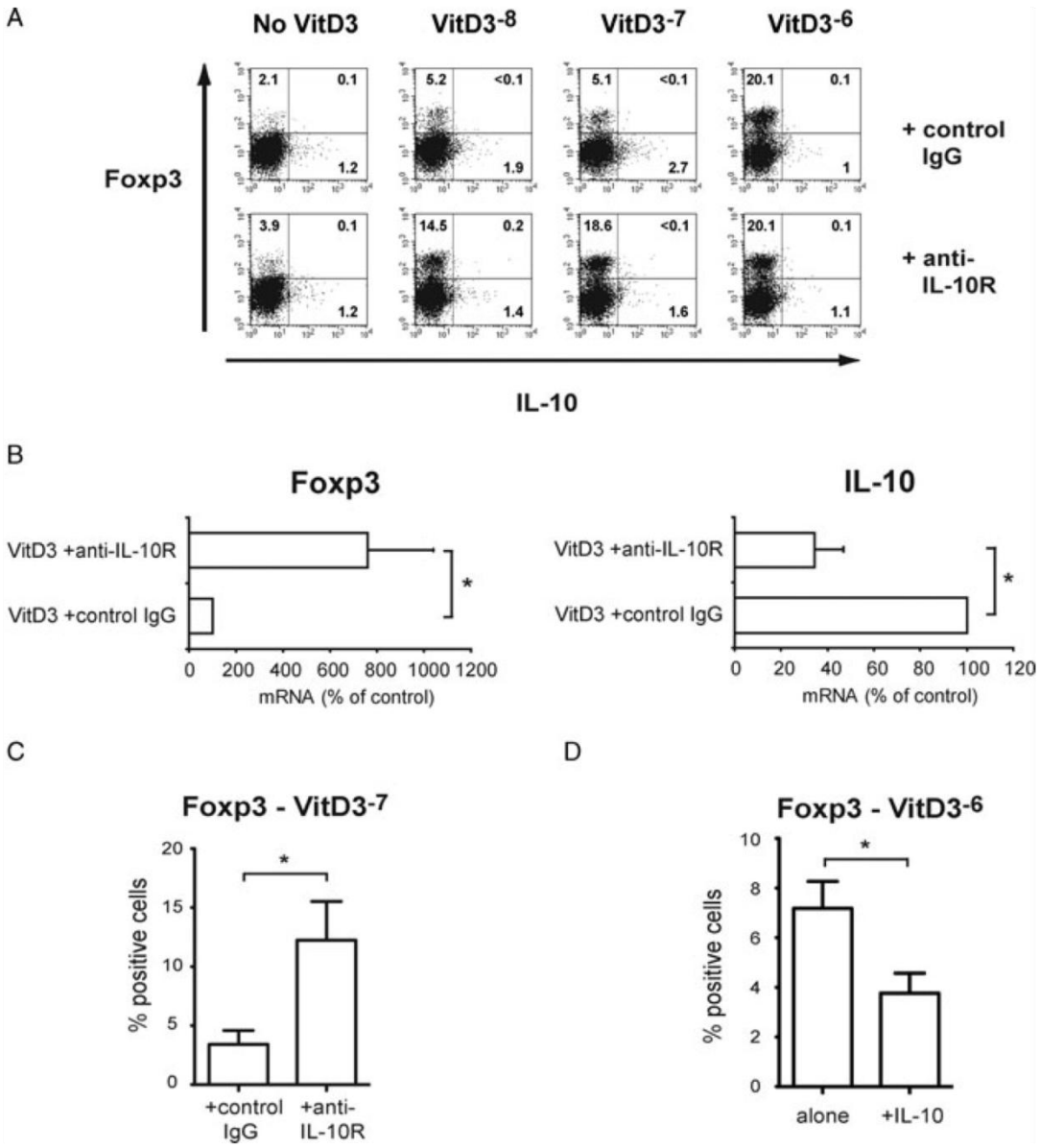

Figure 4. The enhancement of Foxp3 expression by 1α25VitD3 is impaired by IL-10.Human $CD4^+$ T cells were cultured for two 7 day cycles with anti-CD3, IL-2, and IL-4 (No VitD3) or additionally with the indicated concentration of 1α25VitD3 (VitD3; $10\times$ M) in the presence of IL-10, anti-IL-10R, or control IgG, as indicated. (A) At day 14, cells were re-stimulated for 16 h with anti-CD3 and IL-2. $IL\text{-}10^+$ cells were determined using a commercially available IL-10 secretion assay and subsequently stained for intranuclear expression of Foxp3. Values represent the percentage of gated live cells. (B) Foxp3 and IL-10 gene expression, as determined by real time RT-PCR. Data are shown normalized to an endogenous control (18s rRNA) and expressed relative to IgG control-treated cells. Data are shown as mean +SEM from four independent experiments from different healthy donors. (C) $CD4^+$ T cells were cultured with a concentration of 10^{-7} M 1α25VitD3 (VitD3) in the presence of control IgG or anti-IL-10R. Foxp3 expression was determined by intranuclear staining for Foxp3. (D) $CD4^+$ T cells were cultured with a concentration of 10^{-6} M 1α25VitD3 (VitD3) in the presence or absence of IL-10. Foxp3 expression was determined by intranuclear staining for Foxp3. (C and D) Data are shown as mean +SEM of four experiments each performed with an individual donor. $^*p < 0.05$ as determined by the Mann–Whitney rank sum test.

Foxp3 transcripts, with a reciprocal decrease in IL-10 transcripts (Fig. 4B).

To confirm these findings of the effects of IL-10 on 1α25VitD3-enhanced Foxp3 expression, a complimentary approach was used. $CD4^+$ T-cell stimulation cultures were established with high 10^{-6} M 1α25VitD3 in the presence or absence of recombinant IL-10. As predicted, the presence of IL-10 significantly inhibited the frequency of $Foxp3^+$ T cells compared with 10^{-6} M 1α25VitD3 alone (Fig. 4D).

TGF-β is required for the peripheral induction of Foxp3, both alone and in conjunction with retinoic acid (RA) [16–20]. Note in this study, no significant increase in Foxp3 expression was observed when exogenous TGF-β alone was added to cultures containing 10^{-6} M or 10^{-7} M 1α25VitD3 (data not shown).

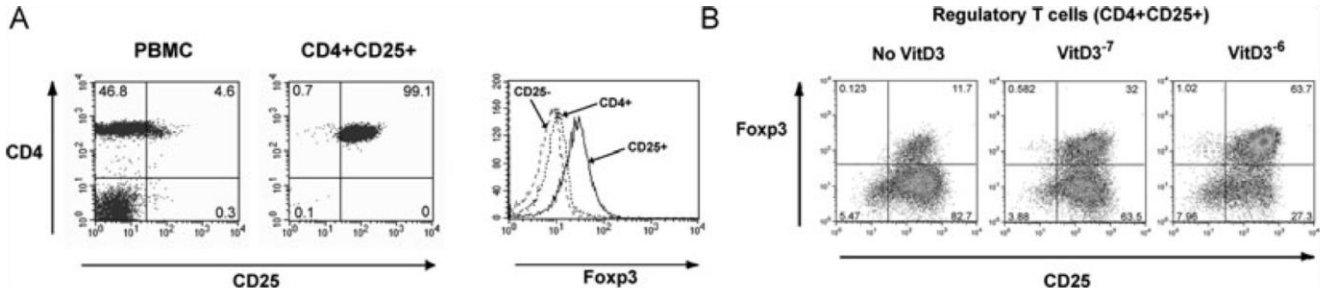

Figure 5. Human $CD4^+CD25^+$ T cells retain Foxp3 expression in the presence of 1α25VitD3. (A) Human $CD4^+CD25^{high}$ Treg cells were sorted from $CD4^+$ T cells by flow cytometry. Foxp3 expression is depicted in the overlay histogram on the right.(B) Sorted $CD4^+CD25^+$ cells were cultured with anti-CD3 and IL-2 (50 U/mL) in the absence (No VitD3) or the presence of 10^{-7} M or 10^{-6} M 1α25VitD3 (VitD3) as indicated for 7 days and then assessed for expression of CD25 and Foxp3 by flow cytometry. Data are representative of three independent experiments each performed with a different healthy donor.

However, neutralization of endogenous TGF-β (by the addition of an antibody specific for TGF-β to the culture) decreased 1α25VitD3-enhanced Foxp3 expression (Supporting Information Fig. 1), suggesting a possible role for TGF-β.

1α25VitD3 maintains the expression of $Foxp3^+$ T cells in culture

Human $CD4^+CD25^{high}$ cells, which are largely $Foxp3^+$, are known to lose expression of Foxp3 over time upon culture in vitro. To determine if 1α25VitD3 acted to maintain the expression of Foxp3 in this population, $CD4^+CD25^{high}$ (>99% $CD25^+$; 86% $Foxp3^+$; Fig. 5A) T cells were isolated by cell sorting and cultured for 7 days with or without 1α25VitD3. The frequency of $Foxp3^+$ cells diminished from 86 to 11.7% upon culture with anti-CD3 and low dose IL-2 alone, shown in a representative plot in Figure 5B. In contrast, stimulation of $CD4^+CD25^{high}$ cells in the presence of 10^{-7} M and 10^{-6} M 1α25VitD3 resulted in 32% and 63.7% of the cells remaining $Foxp3^+$, respectively, in the representative data shown in Fig. 5B. These data suggest 1α25VitD3 contributes to the retention of $Foxp3^+$ expression by human $CD4^+CD25^{high}$ T cells.

To confirm and extend these data, these experiments were repeated with mouse T cells. When total unfractionated $CD4^+$ cells (>99% pure) were cultured in the absence or presence of 1α25VitD3, Foxp3 expression was increased from 3% to 7.3% with 10^{-7} M 1α25VitD3 in the example shown (Supporting Information Fig. 2A). When purified $CD4^+Foxp3GFP^+$ cells (>97% $Foxp3^+$) were stimulated with anti-CD3 and IL-2, in the absence of 1α25VitD3, Foxp3 expression was greatly reduced following 7 days of culture. In contrast, in cultures containing 10^{-7} M and 10^{-6} M 1α25VitD3, more than 50% of the cells remained $Foxp3^+$ (Supporting Information Fig. 2B). The addition of RA plus TGF-β to all cell cultures enhanced Foxp3 expression as predicted from independent published data. Collectively, these data support the evidence from experiments with human T cells that 1α25VitD3 enhances the frequency of $Foxp3^+$ cells by maintaining Foxp3 expression in culture.

1α25VitD3 favors the expansion of $Foxp3^+$ over $Foxp3^-$ T cells

An enrichment in the percentage of $Foxp3^+$ cells was observed in the presence of 10^{-6} M 1α25VitD3, or in the presence of lower concentrations of 1α25VitD3 plus anti IL-10R antibody. As 1α25VitD3 has well-documented inhibitory effects on T-cell cycle and proliferation, we investigated the capacity of 1α25VitD3 to directly modify the proliferation of $Foxp3^+$ versus $Foxp3^-$ T cells using CellTrace Violet. This highly stable dye enabled monitoring of cell division of $Foxp3^+$ and $Foxp3^-$ cells for up to 14 days of culture by flow cytometry.

In the absence of 1α25VitD3, comparable proportions of the major $Foxp3^-$ and the minor $Foxp3^+$ T-cell populations had proliferated by day 7 and day 14 of culture. The addition of 1α25VitD3 10^{-6} M to the culture, impaired both $FoxP3^-$ and $Foxp3^+$ T-cell proliferation at days 7 and 14 (Fig. 6A). However, whereas the $Foxp3^-$ T-cell proliferative response was almost completely abrogated, a clear $Foxp3^+$ T-cell response, albeit reduced, could still be observed. The difference in the proliferative response between these two populations was significant (Fig. 6B). The addition of anti-IL-10R into cultures containing 10^{-7} M 1α25VitD3 resulted in a significant increase in cell division in the $Foxp3^+$, but not the $Foxp3^-$ T cells at day 7 (Supporting Information Fig. 3) and to a lesser extent at day 14 (data not shown). Together these data suggest that a contributory mechanism by which 1α25VitD3 increases the frequency of $Foxp3^+$ cells is via the preferential inhibition of the proliferation of $Foxp3^-$ cells.

In order to distinguish between effects of 1α25VitD3 on existing Treg cells compared with newly generated adaptive and/or activation-dependent Foxp3 expression arising from the effector population, Treg ($CD4^+CD25^{high}CD127^{low}$) and T-effector ($CD4^+CD25^-CD127^{high}$) cells were isolated from the same donor to high purity (Supporting Information Fig. 4). The two populations were individually labeled with CellTrace and then co-cultured at the original ratio (one Treg to nine effector cells), combining either labeled Treg with unlabeled T-effector cells, or conversely labeled T-effector cells with unlabeled Treg cells. These experiments demonstrate that a very low frequency of $Foxp3^+$ T

cells arise from the labeled effector T-cell population, cultured alone or with labeled Treg cells, in the absence or presence of 1α25VitD3 (<2% at day 14; data not shown). These data suggest that 1α25VitD3 is not acting to enhance adaptive/activation-dependent Foxp3 expression. Furthermore, across a dose titration of 1α25VitD3, Treg cell proliferation was only reduced at 10^{-6} M 1α25VitD3, whereas at all other concentrations proliferation was unaffected or even enhanced (Fig. 6C and D). In contrast, proliferation of labeled effector T cells in co-culture was reduced at all concentrations of 1α25VitD3 tested (10^{-9}–10^{-6} M 1α25VitD3; Fig. 6C and D). These data imply that culture of T cells with 1α25VitD3 preferentially expands Treg over T-effector cells.

Evidence for a role of 1α25VitD3 in the maintenance of Foxp3$^+$ T-cell frequencies in vivo

Our earlier studies demonstrated that 1α25VitD3 enhances IL-10 expression by CD4$^+$ T cells not only in culture, but also following ingestion of standard formulary doses of 1α25VitD3 by both steroid refractory asthma patients and healthy subjects [12, 14]. Subsequent work has demonstrated that no parallel increase in Foxp3 gene expression occurred in the same peripheral blood CD3$^+$CD4$^+$ T cells, analyzed directly ex vivo pre- and post-1α25VitD3 ingestion (data not shown). To investigate whether vitamin D might influence Foxp3 expression in the tissues, we analyzed the frequency of CD4$^+$Foxp3$^+$ cells in bronchoalveolar lavage (BAL) samples available from a pediatric severe asthma cohort under study, where serum 25-hydroxyvitamin D3 status was also being assessed (Supporting Information Table 1) [21]. Strikingly the majority of these patients showed a vitamin D status reflecting insufficiency (<75nmol/L) or deficiency (<50 nmol/L) [22]. A statistically significant correlation between serum vitamin D status, and the frequency of CD4$^+$Foxp3$^+$ T cells in the BAL was observed ($r = 0.71, p = 0.02$), suggesting an in vivo correlate of our in vitro observations on the capacity of 1α25VitD3 to influence Foxp3$^+$ Treg cell prevalence (Fig. 7 and Supporting Information Fig. 5).

Discussion

Interest in enhancing Treg cells in patients is clearly driven by the therapeutic potential of these cells. An attractive approach would be the use of pharmacological agents such as 1α25VitD3, or vitamin D supplementation, to induce the expansion and/or maintenance of Treg cells. This approach is especially suited to ongoing chronic diseases such as asthma that occur at high prevalence, where a simple treatment such as vitamin D supplementation would be relatively safe, acceptable to patients, and cost effective. The present study aimed to investigate the capacity of 1α25VitD3 to promote Treg cells and whether dose-dependent limitations exist.

Early indications from clinical studies suggest vitamin D treatment of patients enhances T-cell expression of IL-10 in vivo, although data on the impact on Foxp3$^+$ Treg cell frequencies in human peripheral blood are less clear [12, 23–26]. Here, we demonstrate that the active form of vitamin D3 increases the frequency of both IL-10$^+$ and Foxp3$^+$ cells in cultures of human peripheral blood derived CD4$^+$ T cells. The two Treg cell subsets promoted by 1α25VitD3 are distinct cell populations that are optimally induced by different concentrations of 1α25VitD3 in culture.

Both Foxp3$^+$ and IL-10$^+$ 1α25VitD3-promoted T cells exhibited comparable regulatory activity in a conventional in vitro suppression assay. However, more than one inhibitory mechanism appears to exist. Inhibition by T cells generated under conditions that optimally promoted IL-10 was reversed upon addition of an antibody that blocked IL-10 signaling to the co-culture suppression assay. In contrast, the suppressive activity of Foxp3$^+$ cells, generated in the presence of high-dose 1α25VitD3, was not reversed by neutralization of IL-10. A number of additional mechanisms of suppression by Foxp3$^+$ Treg cells have been reported [27].

To investigate how vitamin D modulates the frequency of Foxp3$^+$ cells in culture, initial studies focused on the capacity of 1α25VitD3 to maintain expression of Foxp3 by existing Treg cells. 1α25VitD3 maintained the levels of Foxp3 expression in human CD4$^+$CD25^{high} Treg cells, which otherwise were lost upon in vitro culture. This observation was reproduced using Foxp3GFP CD4$^+$ cells from reporter mice. Using the CellTrace together with Foxp3 staining, we further demonstrated that 1α25VitD3 allowed the preferential expansion of Foxp3$^+$ T cells over Foxp3$^-$ (effector) T cells and this could provide a contributory or additional mechanism by which 1α25VitD3 promotes Foxp3$^+$ Treg cells. These data, together with earlier studies suggesting that vitamin D increases Foxp3 expression in human naïve T-cell cultures [10, 28], indicate that vitamin D acts through several different mechanisms to enhance Foxp3 expression. IL-2 plays a central role in the maintenance of a functional Treg cell compartment [29, 30]. Interestingly, our data suggest that one mechanism by which 1α25VitD3 may act to maintain Treg cells is via the observed increased expression of the alpha chain of the IL-2 receptor, CD25, and this could be relevant to all of the pathways proposed above.

An unprecedented finding of the present study is the reciprocal regulation of Foxp3 and IL-10 by 1α25VitD3. The phenotype of the Treg cell population generated is likely to depend not only upon the level of vitamin D available, but also the local cytokine milieu. In addition to the well-documented requirement for IL-2 to maintain Treg homeostasis, we envisage that in the presence of higher concentrations of IL-10, IL-10-Treg induction will be favored. Conversely, elevated TGF-β and reduced IL-10 in 1α25VitD3-driven cultures will result in Foxp3$^+$ Treg cell generation. Our observations that exogenous IL-10 in 1α25VitD3-driven cultures reduces the frequency of Foxp3+ T cells, while blocking IL-10 signaling in these cultures increases Foxp3$^+$ T-cell frequency, further indicate reciprocity in control of IL-10 and Foxp3 expression. We show that Foxp3 expression was significantly enhanced by 1α25VitD3 following 14 days of culture (as previously reported for IL-10 [12]), while enhancement at day 7 was variable

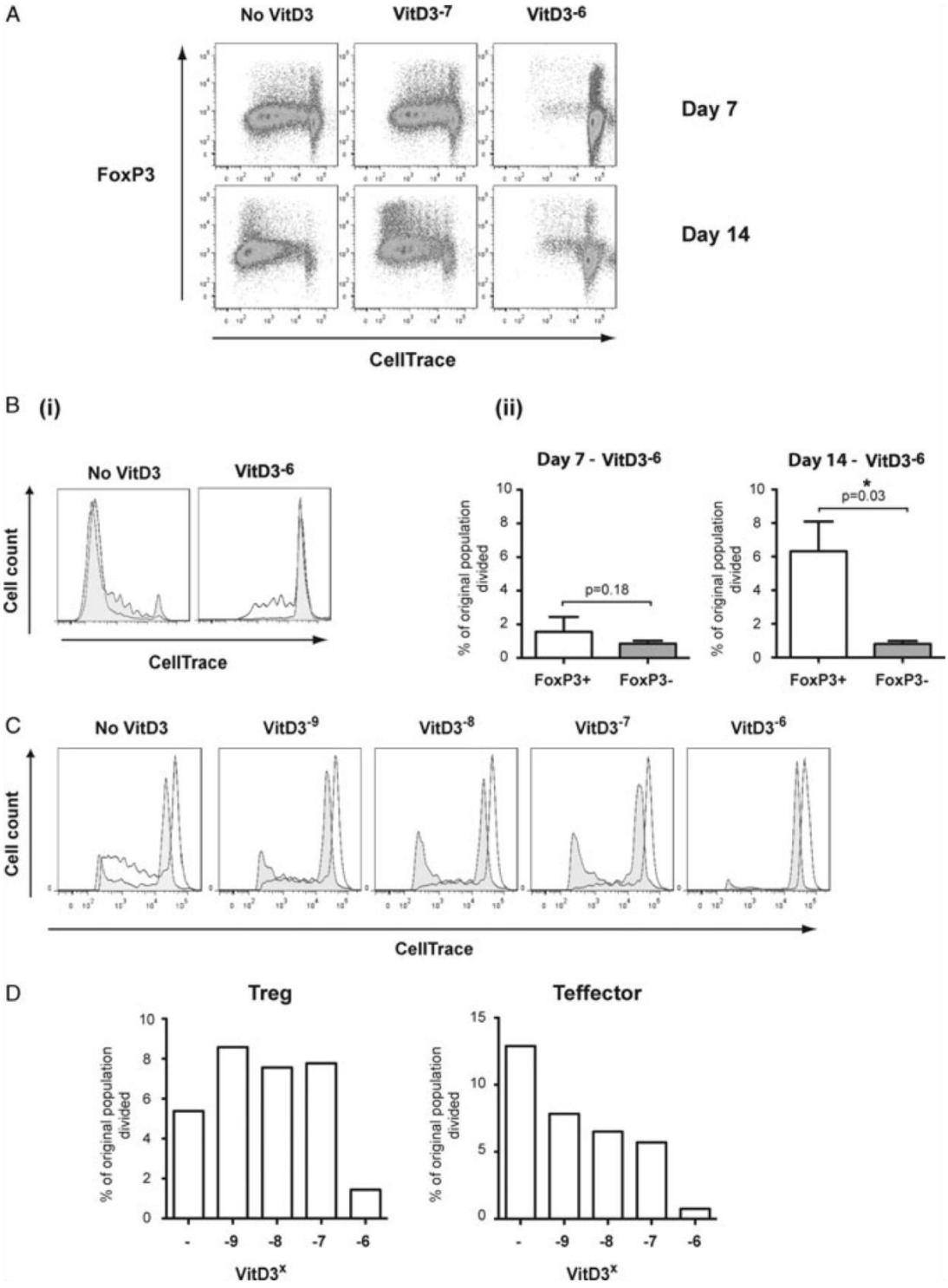

Figure 6. 1α25VitD3 favors the proliferation of Foxp3$^+$ over Foxp3- T cells.(A) Human CD4$^+$ T cells were labeled with CellTrace™ Violet and then cultured for one or two 7-day cycles with anti-CD3, IL-2 (No VitD3), or additionally with the indicated concentration of 1α25VitD3 (VitD3; 10× M). At day 7 and 14, cells were stained for Foxp3 and analyzed by flow cytometry. Proliferation was assessed by loss of expression of CellTrace with each cell division. (B) (i) Representative histograms showing CellTrace expression in Foxp3$^-$ (filled histogram) and Foxp3$^+$ (open histogram) T cells at day 14. Note the peaks represent successive generations of cells. (ii) Data from day 7 ($n = 7$) and day 14 ($n = 4$) of the percentage of original population divided are shown as mean ± SEM of the indicated number of experiments. *$p < 0.05$ as determined by the Mann–Whitney rank sum test. (C) Overlay histograms from a representative donor showing CellTrace expression in individually labeled Treg or T-effector populations that were then co-cultured with the nonlabeled population for 14 days at the original ratio of 1:9 in the absence or presence of 1α25VitD3 as indicated. Filled histograms show proliferation of labeled Treg cells that were co-cultured with unlabeled T-effector cells. Open histograms assess proliferation of labeled T-effector cells, co-cultured with unlabelled Treg cells. (D) Data from (C) presented as percentage of original population divided. Representative data from three independent experiments each performed with different donors are shown.

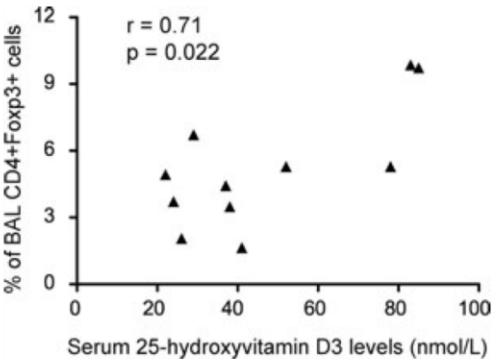

Figure 7. Serum 25-hydroxyvitamin D3 correlates with the frequency of $CD4^+$ $FoxP3^+$ cells in BAL of pediatric asthma patients. Pediatric patients with severe therapy resistant asthma (STRA) were analyzed for the presence of Treg cells (percentage of $CD4^+$ cells expressing Foxp3) in bronchoaveolar lavage fluid. Serum was collected from the same patients and the concentration of the circulating form of vitamin D3, 25-hydroxyvitamin D3 was assessed by two-dimensional high performance liquid chromatography system–tandem mass spectrometry. Each point represents an individual patient from 11 experiments performed.The *r* and *p* values were assessed using Pearson's correlation test.

and did not achieve statistical significance (data not shown). This may indicate that longer-term exposure to vitamin D, arguably reflecting the situation in a vitamin D replete individual, will favor Treg cells in patients.

A high prevalence of vitamin D insufficiency has been documented in asthma cohorts worldwide. A strong association between low vitamin D status with severity and poor control of asthma has been shown by several independent groups of investigators [31–36]. Our own studies have addressed this in a severe therapy-resistant pediatric asthma cohort. We observe highly significant associations between serum 25-hydroxyvitamin D3 levels with lung function, asthma severity, and control [21]. Using this unique patient cohort, we recorded a positive correlation between serum 25-hydroxyvitamin D3 levels with the frequency of $CD25^+Foxp3^+$ T cells in the airways, complimenting our in vitro observations. Additionally, we have very recently observed that the frequency of $CD4^+CD127^{low}Foxp3^+$ T cells in the periphery of steroid sensitive is higher than in steroid refractory adult moderate to severe asthmatics, and go on to demonstrate a significant correlation between serum vitamin D status and the number of these cells in the periphery [37]. Together, these association data support the concept that vitamin D status may control $Foxp3^+$Treg frequencies in vivo, which could represent a mechanism whereby vitamin D treatment dampens asthma symptoms. However, two recently published studies using either a hypocalcaemic vitamin D analogue [24] or high-dose vitamin D supplementation in patients with multiple sclerosis [23] showed no increase in the frequency of peripheral blood $CD4^+Foxp3^+$ T cells following vitamin D treatment. Clearly further translational studies in patients are required to fully understand the impact of vitamin D on Treg cells in humans.

Although these studies were designed to investigate a role for vitamin D in a therapeutic context, they also have implications regarding a physiological role for vitamin D in immune modulation, including Treg frequency as highlighted by the data from pediatric BAL. Extrarenal synthesis of active vitamin D is increasingly being recognized as important for modulation of both innate and adaptive immunity [38]. Vitamin D3 can be metabolized into 1α25VitD3 by a variety of immune and structural cells including DCs [38, 39], macrophages [40], and epithelial cells [41], which is proposed to result in relatively high 1α25VitD3 concentrated locally in the lymphoid microenvironment for presentation to interacting T-cells [42]. Thus, local synthesis of 1α25VitD3 in tissues may influence Treg frequency, although what constitutes "physiological" levels of 1α25VitD3 generated locally in tissues, and how these reflect observations from in vitro studies is as yet difficult to ascertain. Production of 1×10^{-9}–6×10^{-8} M 1α25VitD3 by antigen presenting cells has been reported [39, 42], which is not that dissimilar to what is used in the present study.

In summary, vitamin D deficiency and insufficiency is increasing being associated with a wide range of immune-mediated pathologies [22, 43]. In a translational setting, these data suggest that 1α25VitD3, over a broad concentration range, is likely to be safe and effective in enhancing the frequency of both $Foxp3^+$ and $IL\text{-}10^+$ Treg cell populations in patients. We believe, supported by our data and others, that vitamin D delivered either through supplementation or pharmacologically, including novel derivatives that lack the side effect of hypercalcaemia, could prove candidates for increasing the frequency of Treg cell populations in patients. This type of approach may be particularly amenable in patients where individually tailored therapies are impractical.

Materials and methods

Mice

Wild-type C57BL/6 and genetically modified Foxp3GFP C57BL/6 [44] and TCR transgenic (TCR7) mice on a $Rag1^{-/-}$ background specific for hen egg lysozyme [45] crossed to Foxp3GFP C57BL/6 (Foxp3GFP TCR7 $Rag1^{-/-}$) mice [46] were bred and maintained under specific pathogen-free conditions at NIMR according to the Home Office UK Animals (Scientific Procedures) Act 1986 and used at 8–12 weeks of age.

Human studies

PBMCs were obtained from normal healthy individuals in the majority of experiments. The Ethics Committee at Guy's Hospital approved the study and all donors provided informed consent.

Twelve pediatric patients with severe therapy-resistant asthma were also studied (Supporting Information Table 1). Severe therapy-resistant asthma was defined as persistent chronic symptoms of airway obstruction, despite treatment with high-dose inhaled corticosteroids and trials of add on drugs, and/or recurrent severe asthma exacerbations. All children had been through a

detailed protocol to optimize adherence and other aspects of basic management, as far as possible [21, 47]. Bronchoscopies in the pediatric subjects were performed as previously described [48]. The Royal Brompton Hospital Ethics Committee approved the study; written age-appropriate informed consent was obtained from parents and children. Serum 25-hydroxyvitamin D was measured using a two-dimensional high performance liquid chromatography system–tandem mass spectrometry.

Cell purification and culture

Human PBMCs were isolated as previously described [12]. $CD4^+$ T cells were purified by positive selection using Dynabeads (Invitrogen; typical purity 98.5%) or cell sorting (typical purity 99.5%) using a FACSARIA flow cytometer (Becton Dickinson). $CD4^+CD25^{high}$ (purity >99%) cells were isolated by cell sorting from Buffy coats from the National Blood Service. Treg ($CD4^+CD25^{high}CD127^{low}$; typical purity >98%) and effector T cells ($CD4^+CD25\text{-}CD127^+$; purity >99%) were cell sorted from cones obtained from the National Blood Service.

Human $CD4^+$ T cells (1×10^6 cells/mL) were stimulated with plate-bound anti-CD3 (1 μg/mL; OKT-3) in RPMI containing 50 U/mL recombinant hIL-2 (Eurocetus), 10 ng/mL hIL-4 (NBS), and calcitriol (1α25VitD3; BIOMOL Research Labs) as indicated, for 7 day cycles. In some experiments, 5 ng/mL IL-10 (R&D), 5 μg/mL anti-TGF-β (clone 1D11; R&D), 5 μg/mL anti-IL-10R (clone 3F9-2; BD-Pharmingen), or the appropriate isotype control antibody were added, as indicated. Note cells used for proliferation analysis were stained at day 0 with 5 mM CellTrace™ Violet (Invitrogen), according to manufacturers' instructions.

Murine $CD4^+$ T cells were FACS sorted on a MoFlo cytometer (Beckman Coulter) for $CD4^+$ (purity >99%), $CD4^+CD44^{low}CD25^-$ ($Foxp3GFP^-$; purity >99%), or $CD4^+Foxp3GFP^+$ (purity >97%) from CD4-enriched spleen cells. Cells were stimulated in flat-bottom 96-well plates (0.25×10^6 cells/mL) with plate-bound anti-CD3 (145-2C11) at 2.5 mg/mL in cRPMI medium [45] containing 5 ng/mL recombinant mIL-2 (Insight Biotechnology) for 7 days. Cells were fed with IL-2 on day 3. Where indicated, 1α25VitD3, 5 ng/mL recombinant hTGF-b1 (Insight Biotechnology), and 10 nM all trans RA (Sigma-Aldrich) were added to T-cell cultures.

Functional assays of regulatory function

$CD4^+$ T-cell lines were generated as described above. $CD4^+CD45RA^+$ naïve T cells were labeled with 2 μM CFSE (Molecular Probes, Eugene) and co-cultured with the autologous line at the ratios indicated, with 0.1 μg/mL plate-bound anti-CD3 and 1 μg/mL anti-CD28 (clone 15E8; Sanquin). In some experiments, anti-IL-10R or IgG control was added to the co-culture. On day 5, cells were stained with propidium iodide (PI; Sigma-Aldrich) for dead cell exclusion and 30,000 CFSE positive viable responder cells were acquired on a FACSCaliber flow cytometer (Becton Dickinson).

Analysis of IL-10 and Foxp3 expression by flow cytometry

Human $IL\text{-}10^+$ cells were identified using a commercially available IL-10 Secretion Assay Detection Kit (Miltenyi Biotec). Foxp3 (clone PCH101) expression was determined by cell staining using the Foxp3 staining buffer set from Ebiosciences. Quadrant markers were set according to the matched isotype control antibody staining. Antibodies used for cell surface phenotyping (BD Biosciences) were PD-1 (clone MIH4), CTLA-4 (clone BN13), CD62L (clone DREG-56), CD25 (clone M-A251), GITR (clone 110416), and CD38 (clone HIT2).

Expression of Foxp3 in murine $CD4^+$ T cells was determined by excluding dead cells with LIVE/DEAD Fixable Red Dead Cell Staining Kit (Invitrogen) and intracellular staining for Foxp3 with staining buffer set from eBiosciences. Samples were acquired on LSR II (BD) flow cytometer.

Real time PCR

RNA was extracted from cell pellets using RNeasy Mini kit (Qiagen). RNA was reverse transcribed using random hexamer primers (Fermantas Life Sciences). Real time (RT) PCR was performed in triplicate using FAM-labeled Assay-on-Demand reagent sets for IL-10 (Hs00174086_m1) and Foxp3 (Hs00203958_m1). RT-PCR reactions were multiplexed using VIC-labeled 18S primers and probes (Hs99999901_s1) as an endogenous control and analyzed using SDS software version 2.1 (Applied Biosystems), according to the $2^{-(\Delta\Delta Ct)}$ method.

Statistics

Results are presented as mean ± SEM, unless indicated. Data were assessed for normality and equal variation after which the appropriate parametric or nonparametric test was performed (see individual figure legends). Differences were considered significant at the 95% confidence level. Correlations were verified with the Pearson's correlation test or the Spearman's rank correlation coefficient, as indicated in the figure legend.

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Abbreviations: **IL-10-Treg**: IL-10-secreting Treg cell · **RA**: retinoic acid · **1α25VitD3**: 1α,25-dihydroxyvitamin D3

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